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MiR-30 suppresses lung cancer cell 95D epithelial mesenchymal transition and invasion through targeted regulating Snail

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Abstract. – OBJECTIVE: As an important factor regulating the epithelial mesenchymal transition (EMT) Snail is associated with lung cancer. Bioinformatics analysis showed that microRNA-30a (miR-30a) may target the 3'-UTR of Snail mRNA. It was exhibited that miR-30a down-regulation was related to tumor size, TNM stage, and poor prognosis of non-small cell lung cancer (NSCLC) patients, which suggests that miR-30a might participate in NSCLC attack. This ... aims to explore the role of miR-30a and S... NSCLC invasion and metastasis.

PATIENTS AND METHODS: NSCLC tumor ... para-carcinoma tissues were collected from ... patients to evaluate the miR-3... Snail e... pressions. The targeted re... betwee... miR-30a and Snail was ver... by us... dual-luciferase reporter assay. ...cells w... ultured in vitro and transfect... w... iR... small interfere RN... getin... il (si-Snail). The expression o... R-30a, S... EMT-related factors, malign... th, invasio... d apoptosis, were com... ed.

RESULTS: Snail was ...ificantly up-regulated, while ...-30a was s...cantly reduced in NSCLC ...sue. MiR-30a st...essed Snail expres... by tar...ing the 3'-UTR of Snail mRNA. 95... ls ex...ted significantly higher Snail, N-ca... nd vim...in levels, while lower ...R-30... adheri... nd occludin expressions ...com... d w... 5C cells. 95D cells presented ...onger ...ant growth and invasive ability ...hereas l...er background apoptosis than ...mimic and/or si-Snail transfection ...ignific... enhanced E-cadherin and occludin ...xpression, while significantly declined N-cad...in and vimentin levels, thus weakening ma...t growth and invasion and increasing cell apoptosis.

CONCLUSIONS: Snail up-regulated, while miR-30a declined in NSCLC tissue. MiR-30a may suppress Snail expression, restrain EMT, and inhibit lung cancer cell invasion.

Key Words:
miR-... Snail, EMT, L... ancer, Invasion.

Introduction

...mall ... lung cancer (NSCLC) is the most ... and main pathological type in lung ...ancer, accounting for about 80-85% of all ca... present, although the imaging and sputum ...y... analysis achieved great progress, the early diagnosis and detection rate of NSCLC is still low because of unapparent early symptoms and lack of sensitive specific indicators. Most of lung cancer patients are in advanced stage when diagnosed, therefore, missing the optimal treatment opportunity. Though biological therapy, immune therapy, and individualized treatment based on the molecular level greatly improve, it still cannot effectively reduce the mortality of patients, leading to the extremely low survival rate. The 5-year survival rate of lung cancer patients is only 15-20%, in which stage I is 60-80%, and stage IV is only 1%[1]. Therefore, exploring the pathogenesis of lung cancer and searching for sensitive and specific markers for early diagnosis, targeted therapy, and prognostic judgment are of great significance to improve early diagnosis, treatment effect, and prognosis. Metastasis and recurrence often occur in lung cancer patients after resection, which are the important reasons to limit the treatment effect. It was confirmed that epithelial mesenchymal transition (EMT) is the start step of tumor invasion and metastasis, which is closely related to tumor metastasis, recurrence, and poor prognosis[2]. MicroRNAs is a kind of endogenous single-stranded non-coding RNAs in eukaryotes at the length

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of 21-24 nucleotides. They can complementary bind to the 3'-UTR of mRNA to degrade mRNA or inhibit translation. It was reported to participate in EMT process and closely related to lung cancer invasion and metastasis[3,4]. Recent studies showed that miR-30a down-regulation was related to tumor size, lymph node metastasis, TNM stage, and poor prognosis of NSCLC patients, indicating that miR-30a may be involved in NSCLC incidence, whereas the specific mechanism is still unclear[5]. Snail is an important regulatory factor in EMT process by up-regulating vimentin and declining E-cadherin to promote EMT. It was revealed that Snail obviously increased in NSCLC patients and was associated with tumor progress, metastasis, and poor prognosis[6]. Bioinformatics analysis showed that miR-30a had good targeted complementary relationship with 3'-UTR of Snail. This study investigated the role of miR-30a and Snail abnormal expressions in lung cancer invasion and metastasis.

Patients and Methods

Patients

A total of 46 NSCLC patients were diag d and received treatments in The Second Affili Hospital, (Kunming, Yunnan, China) between bruary 2015 and March 2016. P ere enro led, including 33 males and 1 male th mean age at 52.9 ± 12.5 years o o patie received radio or chemotherapy bef rge 16 cases in stage I-II case e III, and 10 cases in stage IV, or tissue ara-carcinoma tissue at le from the margin, were obtained ring rgery. The xperiment was appro by the Eth ommittee of Second Affiliat ospital, Kunmi edical University (Ku g, Yun China) and all subjects signed inf d con

ior ents Materials

man static NSCLC cell line 95D an w meta c NSCLC cell line 95C were p from ATCC cell bank (Manassas, VA, A). -1640, fetal bovine serum (FBS), and enicillin-streptomycin were bought from Hyclo- Logan, UT, USA). Lipofectamine 2000 was g om Invitrogen Life Technologies (Carlsbad, CA, USA). RNA extraction kit was purchased from Omega Bio-Tek Inc. (Norcross, GA, USA). Reverse transcription Kit ReverTra Ace qPCR RT Kit was obtained from Toyobo Co. Ltd. (Osaka, Japan). SYBR Real-time PCR Master Mixes was got from Invitrogen Life Technologies (Carlsbad, CA, USA). MiR-30a nucleotide fragments and PCR primers were synthetized by RiboBio (Guangzhou, China). Mouse anti-human Snail and rabbit anti-human E-cadherin antibo obtained from Santa Cruz Biotechn (Santa Cruz, CA, USA). Mouse anti-hum -cadherin and rabbit anti-human vimentin an s were obtained from Cell Signali Techn Inc. (Beverly, MA, USA). BC protein qua tion kit was bought from ster Bi nc. (W China). Annexin V/PI tosis ction kit was from Beyotime (Be g, ransw am-ber was got fro ipore rica, USA). Matrigel wa ined from osciences (Franklin L , USA) whils al-Luciferase® Report Assay em and pGL3-promoter plasm purchase m Promega (Madison, W A).

Culture

O and 95C s were maintained in RPMI-164 dium ining 10% fetal bovine serum (FBS) penicillin-streptomycin and cultured in 37°C and 5% CO_2. Cells in logarithmic were used for experiments.

Luciferase Reporter Gene Vector Construction

The 3'-UTR of Snail gene were amplified based on HEK293 cell genome using the primers as 5′-AATATATAAATTAACTGCTTTATTG-3′ and 5′-CCCTCGAGGCT CCCTCTTCCTCTCC-3′. PCR product was recycled and double digested with XbaI/NotI. Then, it was connected to luciferase reporter vector pGL-3M to transform DH5α competent cells. After colony PCR, the positive clone was screened and the plasmid with correct sequence was applied for cell transfection.

Luciferase Reporter Gene Assay

HEK293 cells in logarithmic phase were seeded in 24-well plate at $4\times10^4/cm^2$ density. When the density reached 50-60%, cells were transfected with 200 ng pGL3-Snail-3'UTR, 50 nmol microRNA nucleotide fragment, and 50 ng pRL-TK mixture mediated by Lipofectamine 2000. After incubated for 4-6 h, Opti-MEM medium was changed to Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin for another 48 h. After cells were washed with PBS for twice, they were added tp 100 µl passive lysis buffer (PLB)

for 15 min to crack. The lysis was moved to a new Ep tube and centrifuged at 10,000 × g for 5 min. The supernatant was added with 50 µl luciferase substrate in luciferase assay buffer II and tested immediately in chemiluminescence apparatus. Then the solution was added with 50 µl Stop & Glo solution to test marine coelenteron luciferase activity in microplate reader. The ratio of luciferase activity and marine coelenteron luciferase activity was treated as relative expression level of report gene.

Cell Transfection

Human Snail gene sequence was used as the template to design and synthetize Snail siRNA interference sequence, si-Snail forward, 5′-GCUGCAGGACUCUAAUCCA -3′, si-XIAP reverse, 5′-UGGAUUAGAGUCCUGCAGC-3′. Meanwhile, negative control was designed as follows. Si-NC forward, 5′-UUCUCCGAACGUGUCACGU TT-3′, si-NC reverse, 5′-ACGUGACACGUUCGGAGAATT-3′. Cells were divided into five groups, including mimic NC group, miR-30a mimic group, si-NC group, si-Snail group, and miR-30a mimic + si-Snail group. Cells were collected at 72 h after transfection for following experiments.

qRT-PCR

Total RNA was extracted with kit from Omega. Cells were added with 0 µl K buffer and 350 µl 70% ethanol. After blending, the solution was moved to the filter column, centrifuged at 10,000 r/min for 1 min, the membrane was washed with 500 µl wash buffer I for 1 time and with 500 µl buffer II for 2 times; then, it was air-dried at room temperature and 3050 µl RNase free water were added for 2 min. The solved RNA was moved to an Eppendorf (EP) tube; after that, was centrifuged at 10000 r/min for 1 min. The reverse transcription system in 20 µl contained 2 µl RNA, 4 µl RT buffer (5×), 1 µl oligo (Random primer mix, 1 µl RT Enzyme Mix, 1 µl RNase inhibitor, and RNase free H_2O. The reverse transcription reaction was performed at 37°C for 15 min and 98°C for 15 min. The obtained cDNA was stored at -20°C refrigerator. PCR amplification was performed using cDNA as template under the effect of TaqDNA polymerase. The PCR primers used were as follows:
miR-497P_{RT}: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACACAAA-3′,
miR-30aP_F: 5′-CTTTCAGTCGGAGTTTGCAGC-3′,
miR-30aP_R:5′-TCAAGTACCCACAGTGCGGT-3′;
U6P_F: 5′-ATTGGAACGATACA GAGAAGATT-3′,
U6P_R: 5′-GGAACGCTTCACGAATTTG-3′;
SnailP$_F$: 5′-ACCCCACATCCTTCTCACTG-3′,
SnailP$_R$: 5′-TACAAAAACCCACGCAGACA-3′; E-cadherinP$_F$: 5′-ATTTTTCCCTCGACACCCGAT-3′, E-cadherinP$_R$: 5′-TCCGTAGACCAAGA-3′; N-cadherinP[illegible]-AGCCAACCTTAACTGAGGAGT-3′, [illegible]adherinP$_R$: 5′-GGCAAGTTGATTGGAGGGATG[illegible] vimentinP$_F$: 5′-GACGCCATCAAC[illegible]GAG[illegible] vimentinP$_R$: 5′-CTTTGTCG[illegible]GTTAGCT[illegible]′, occludinP$_F$: 5′-GA[illegible]CAGG[illegible]GCCTCTAC-3′, occludinP$_R$: [illegible]CAG[illegible]TGTAGTCAGTCTCA-3′; β-actinP[illegible]CTAA[illegible]CCA-AC-3′, β-actinP[illegible]GTCA[illegible]ACGA[illegible]CC-3′. The PCR reaction system was [illegible] of 4.5 µl 2×SYBR [illegible], 1 µl for[illegible] and reverse primer at 2.5 µM, 1 µl [illegible]NA, and 3 µl ddH_2O. The reaction was performed [illegible]BI 7500 at 40 cycles of 95[illegible], 60°C for 30 s, [illegible]74°C for 30 s.

Western Blot

[illegible] protein was extracted using lysis and quantified [illegible]BCA. A total of 60 µg sample was separated [illegible]um dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h and transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk at room temperature for 1 h, then it was incubated in primary antibody at 4°C overnight and was washed with phosphate buffer saline-Tween 20 (PBST) for three times. After, the membrane was incubated in horseradish peroxidase (HRP) labeled secondary antibody at room temperature for 60 min and washed with PBST for three times. At last, the membrane was treated by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) reagent and developed. The data was analyzed using Quantity One software Bio-Rad Laboratories (Hercules, CA, USA).

Clone Formation Assay

Cells in logarithmic phase were digested with 0.25% trypsin and re-suspended in RPMI-1640 medium containing 10% FBS. Cells were then seeded in 10 cm dish at the density of 100 cells/dish within 10 ml medium and cultured at 37°C and 5% CO_2 for 14-21 days. The cultivation was stopped when the macroscopic clone appeared. Next, cells were fixed by 4% paraformaldehyde at room temperature for 15 min and were stained by Giemsa for 20 min. At last, cells were observed under the microscope (4×) to count the clone

number with more than 10 cells. Clone formation rate = (clone number/seeded number) × 100%.

Flow Cytometry

Cells were collected and re-suspended in 195 µl binding buffer. The cell apoptosis was also examined by using the Annexin V/PI apoptosis detection kit in this study. Briefly, cells were incubated with 5 µl Annexin V-FITC and 10 µl PI at room temperature for 15 min in the dark. At last, cells were put on ice for flow cytometry detection.

Transwell Assay

A total of 100 µl matrigel was put on the surface of 8 µm membrane and incubated in 37°C for 30 min. A total of 200 µl cell suspension at 1×10^6/ml were seeded in the upper chamber, while 600 µl RPMI-1640 complete medium containing 10% FBS were added to the lower chamber. After 48 h incubation, the membrane was wiped by sterile swab to remove cells did not pass through the matrigel. Then, the membrane was fixed by 4% paraformaldehyde at room temperature for 30 min and stained by 0.1% crystal violet for 30 min. At last, the membrane was observed under the micr pe. Five views were randomly selected t the cells pass through the membrane.

Statistical Analysis

All data analysis was perfor PSS 18. software (SPSS Inc., Chicag , US The measurement data was prese as mean standard deviation and compared by *t*-test. A $p<0.05$ was depicted as statistical significance.

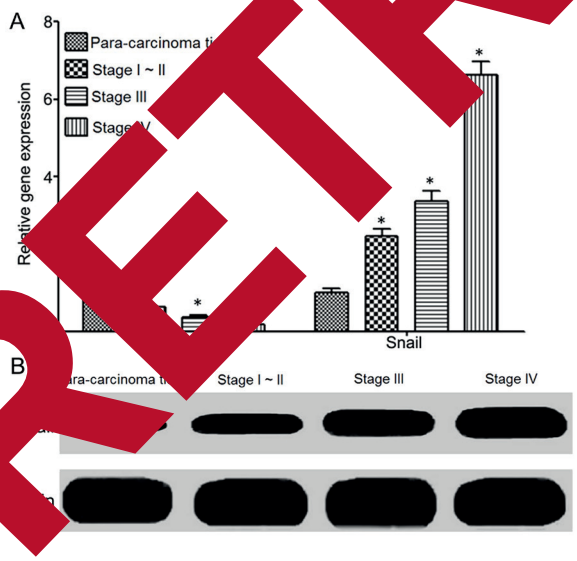

Figure 1. MiR-30a declined, while Snail upregulated in lung cancer tissue. ***(A)*** qRT-PCR detection of miR-30a and Snail mRNA expression. ***(B)*** Western blot detection of protein expression. $^*p<0.05$, *vs.* para-carcinoma tissue.

Results

MiR-30a was Declined and Sna as up-Regulated in Lung Cance ue

Compared with para-carcinoma Snail mRNA and protein expressi signi in-creased in tumor tissue fro SCLC pati lowing TNM upstage (Fi e 1A, It sugg that Snail elevation m inv in lung cancer attack. MiR-30 ev iously uced in tumor tissue pared para inoma tissue. More expressed later stage (Figure ation analy evealed that miR-30a w s signi ly negatively correlated with S RNA ($r=$ 5, $p=0.033$). MiR-30a ma gatively regulate il to participate in l cancer occurrence, invasion, and metastasis.

S was Reg ted by miR-30a

L rase ter gene assay revealed that miR-3 ation obviously reduced the relative luciferase activity of HEK293 cell lysate, while 0a inhibition markedly increased the relative e activity (Figure 2A), indicating that miR-30a can target on the 3'-UTR of Snail mRNA and regulate its expression. Further detection demonstrated that miR-30a up-regulation or reduction significantly declined or elevated Snail expression in 95D cells, respectively. It confirmed that Snail was the target gene of miR-30a (Figure 2B, C, D).

MiR-30a and Snail Expressions Relate to Cell Invasion

qRT-PCR demonstrated that Snail expression was obviously higher, while miR-30a level was lower in human high metastatic NSCLC cell line 95D compared with low metastatic NSCLC cell line 95C (Figure 3A). Western blot presented that E-cadherin and occludin levels were higher in 95D than 95C, whereas N-cadherin and vimentin expressions were lower (Figure 3B). Also, 95D cells exhibited stronger malignant growth and invasion, while lower background apoptosis than 95C cells (Figure 3C, D, and E).

MiR-30a Regulates 95D Cell Malignant Growth, Invasion, and Apoptosis Through Targeting Snail

MiR-30a mimic and /or si-Snail significantly reduced Snail expression in 95D cells (Figure 4A,

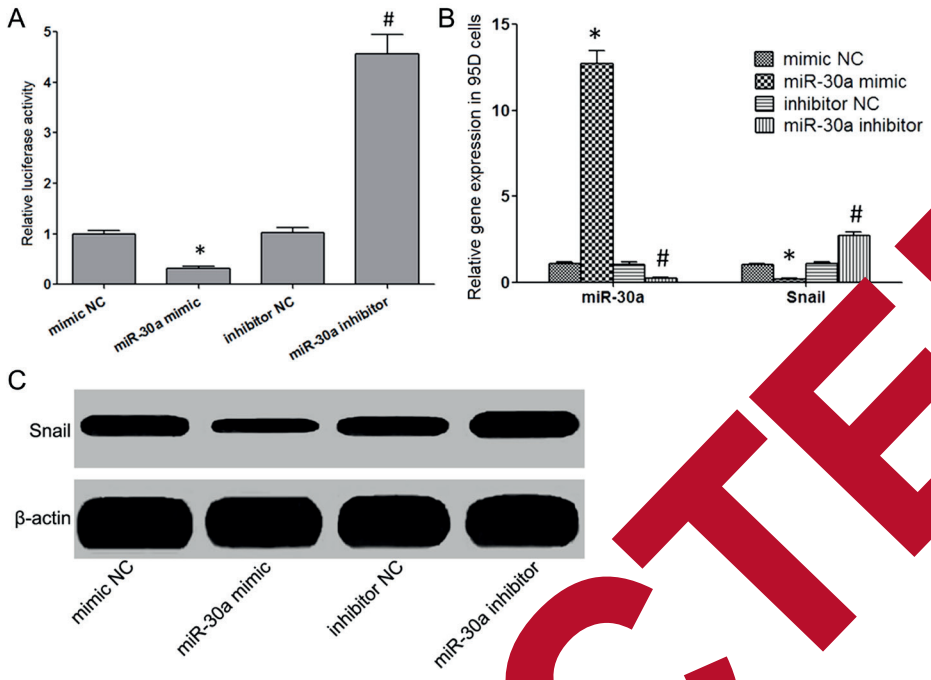

Figure 2. Snail was targeted regulated by miR-30a. ***(A)*** Dual luc[illegible] reporter as[illegible] ***(B)*** qRT-PCR detection of miR-30a and Snail mRNA expression in 95D cells. ***(C)*** Western blot detecti[illegible]nail prot[illegible]xpression in 95D cells. $^{*}p<0.05$, *vs.* mimic NC. $^{\#}p<0.05$, *vs.* inhibitor NC.

B), weakened clone formation (Figure 4C), d[illegible]-30a elevation and/or Snail down-regulated cell invasion (Figure 4E), and enhanced [illegible]tion obviously reduced N-cadherin and Vimentin apoptosis (Figure 4D). Furthe[illegible] showe[illegible] expression, while increased E-cadherin and occlu-

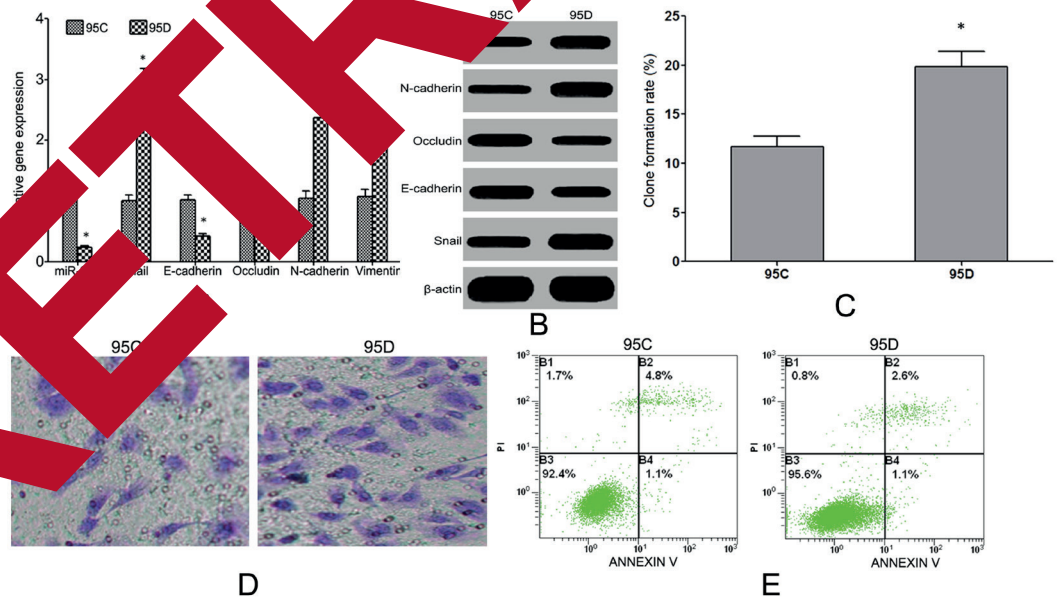

Figure 3. MiR-30a and Snail expressions were related to cell invasion. ***(A)*** qRT-PCR detection of gene expression. ***(B)*** Western blot detection of protein expression. ***(C)*** Clone formation assay determination of malignant growth. ***(D)*** Transwell assay detection of cell invasion. ***(E)*** Flow cytometry detection of cell apoptosis. $^{*}p<0.05$, *vs.* 95C.

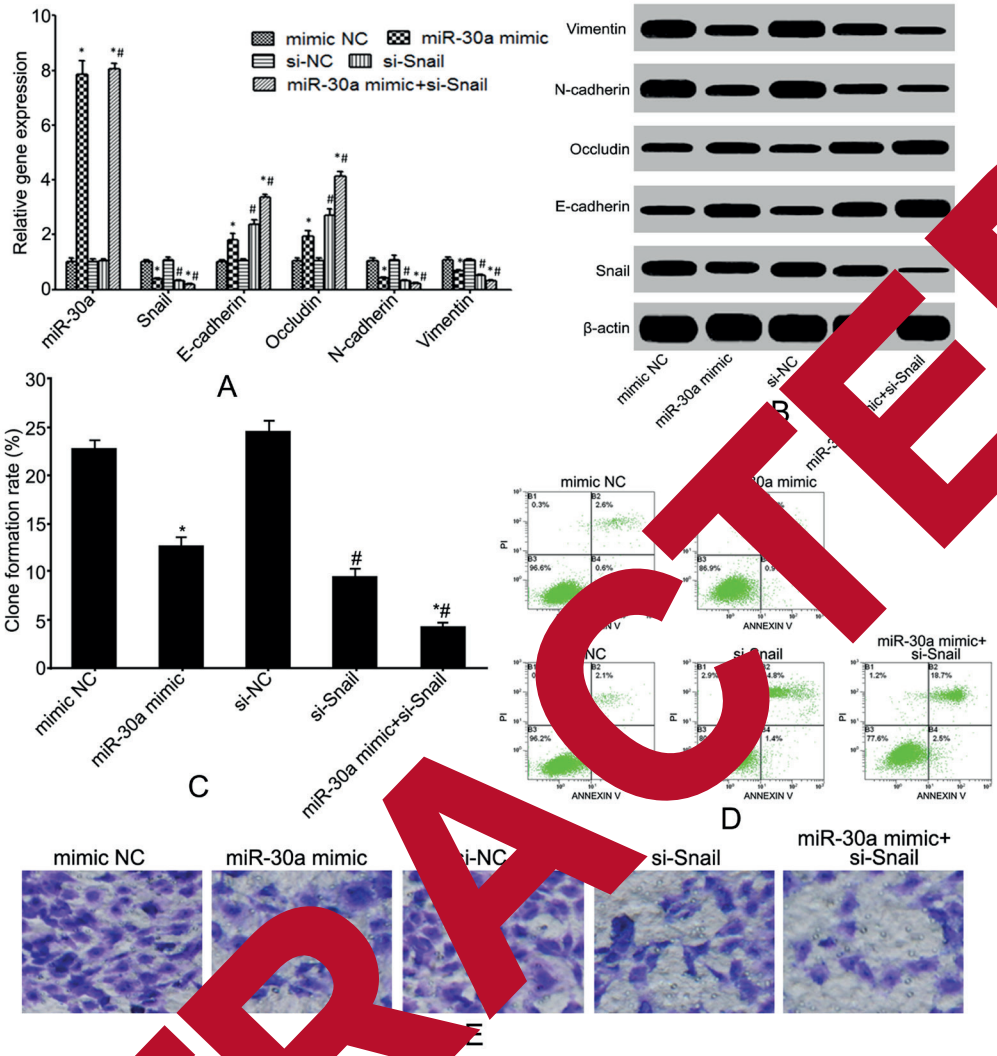

Figure 4. MiR-30a r ed 95D lignant growth, invasion, and apoptosis through targeting Snail. ***(A)*** qRT-PCR detection of gene ex n. ***(B)*** Weste t detection of protein expression. ***(C)*** Clone formation assay determination of malignant growth tometry det of cell apoptosis. ***(E)*** Transwell assay detection of cell invasion. $^{*}p<0.05$, *vs.* mimic NC. $^{\#}p<0.$ *vs.* in NC.

di ls in 9 (Figure 4A, B). It suggested that miR- Snail rmal expressions were ciat h lun cer malignant growth, in- , and .

Discussion

ung cancer is one of the largest malignant t s to human health and life with the fastest growing morbidity and mortality[7]. It was estimated more than 1.8 million new diagnosed lung cancer patients and 1.59 million died of lung cancer worldwide in 2012, accounting for 12.9% and 19.4% of all cancer patients, respectively[8]. The prevalence ratio of lung cancer between men and women is about 3:1-5: 1. Lung cancer morbidity and mortality account for the first in male and second in female, among all malignant tumors[9], thus becoming the leading cause of cancer death around the world. In recent years, the morbidity and mortality of lung cancer significantly increased in China. The number of lung cancer patients increased from 606 thousand to 733 thousand, while the prevalence rate elevated from 46.1/100,000 to 50.4/100,000, and the fatality rate raised from 37.0/100,000 to 42.3/100,000 in recent 5 years[10]. Though the new anti-cancer drugs and treatment measures kept

emerging, the clinical curative effect and prognosis of lung cancer show no obviously improvement[11]. Most of patients are in the middle-late stage or with metastasis because of the unapparent symptoms in early stage, resulting in the low overall survival rate[12]. Therefore, elucidating the pathogenesis of lung cancer, searching specific molecular markers, and exploring the new diagnosis and treatment strategy, are of great significance to improve survival rate and prognosis of lung cancer patients. Lung cancer invasion and metastasis is a complex, multi-stage, and multi-factor regulated biological process, including lung cancer cells detach from the primary site, enter the surrounding stroma, and finally migrate to distance and form new metastatic lesion via blood or lymphatic vasculature[13,14]. EMT refers to the biological process of epithelial cells transform into mesenchymal phenotype cells through specific program, which plays a critical role in embryonic development[15], tissue remodeling, and tumor metastasis[16], and organ fibrosis[17]. It is mainly featured as E-cadherin and occludin reduction, N-cadherin elevation, cytokeratin skeleton transform to vimentin, and the morphology of mesenchymal cells[18]. EMT is the initial ste[illegible] an important biological process of lung canc[illegible] source from which epithelial cells obtain mig[illegible]n and invasion ability. In the EMT process, epith[illegible] cells lose the cell polarity, cell - basement me[illegible] brane, and cell - cell adhesion[illegible] increas[illegible] migration and athletic abilit[illegible]us o[illegible]ning the mesenchymal phenotype a[illegible]n migra[illegible] and invasion, anti-apoptosis, [illegible]nd [illegible]adi[illegible] lar matrix[19]. Recent [illegible]es s[illegible]hat miR-[illegible]0a down-regulation w[illegible]osely asso[illegible]d with tumor size, lymph nod[illegible]sis, TNM [illegible]and poor prognosis of [illegible] canc[illegible]ggesting t[illegible]t miR-30a may partic[illegible]e in the pat[illegible]esis of lung cancer[5]. Our stu[illegible]evealed that mi[illegible]a level obviously decli[illegible]n lung[illegible]ncer tissue[illegible]ompared with para-[illegible]oma[illegible]e, and further reduced following upstag[illegible]eated th[illegible]iR-30a may be involved [illegible]ung [illegible] occu[illegible]ce and related to lung cancer invasio[illegible]astasis, which was in accordan[illegible]with Ta[illegible] al[5] report. Snail is an important r[illegible] factor in EMT process. It can suppress [illegible]adhe[illegible]gene transcription and expression by [illegible]nding with its promoter E-box to promote EMT [illegible] tumor metastasis[20]. Also, Snail can increase N[illegible]adherin expression to facilitate EMT[21]. Grant et al[6] demonstrated that Snail upregulated in NSCLC patients, which was associated with tumor progression, metastasis, and poor prognosis[6]. Hung et al[22] revealed that Snail significantly increased in NSCLC tumor tissue and was correlated with survival rate. Our results exhibited that Snail expression in lung cancer tissue was apparently higher than para-carcinoma tissue, and elevated following TNM upstage, which was in agreement with Grant et al[6] study. Bioinformatics analysis sh[illegible] miR-30a had good targeted complen[illegible]ry rela[illegible] tionship with the 3'-UTR of Snail. [illegible]luciferase reporter assay presented that miR-3[illegible]mic and inhibitor apparently decline[illegible]d en[illegible] the luciferase activity in HEK2[illegible]ell lysate, [illegible]ti[illegible] vely. Moreover, they sig[illegible]antly r[illegible]ce and[illegible] vate Snail mRNA and[illegible]ein e[illegible]ssion in 95D cells, confirming t[illegible]S[illegible]the tar[illegible]gene of miR-30a. Th[illegible]re, this[illegible] furth[illegible]nvestigated the role[illegible]iR-30a and[illegible]regulated expression [illegible]ng[illegible]cer invasio[illegible]d metastasis. It was found that 9[illegible]lls presented stronger invasive[illegible] and mar[illegible]t growth, lower miR-30[illegible]higher Snail than[illegible] cells, revealing that [illegible]30a down-regulation induced Snail elevation [illegible]be related t[illegible]g cancer cell invasion enhanc[illegible]t. In addit[illegible] we observed that 95D exhibite[illegible]er ba[illegible]ound apoptosis than 95C cells, which[illegible]ated to the apoptosis reduction during EMT enhancement process[23]. Furthermore, we [illegible]d the impact of miR-39a and Snail on 95D [illegible]ell g[illegible]wth, invasion, and apoptosis. The results showed that the enhanced miR-30a and/or inhibition of Snail significantly suppressed N-cadherin and Vimentin expression, while elevated E-cadherin and occludin levels in 95D cells, thus to restrain cell malignant growth and EMT. Franco et al[24] observed that Snail enhancement promoted EMT induced by TGF-β and inhibited tumor cell apoptosis. Wan et al[25] demonstrated that downregulation of Snail expression increased the sensitivity of liver cancer cells to apoptosis induced by TNF related apoptosis inducing ligand (TRAIL), revealing the effect of Snail in apoptosis antagonism. This study also found that miR-30a upregulation and/or Snail suppression promoted 95D cell apoptosis, which may be associated with the regulatory role of Snail in apoptosis. It may have the same mechanism with the report of Franco et al[24] and Wan et al[25], whereas the specific mechanism is still unclear.

Conclusions

Snail was abnormally upregulated, while miR-30a was declined in lung cancer tissue. MiR-30a may restrain EMT and lung cancer invasion by targeted suppressing Snail expression.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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