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MiR-22 inhibits lung cancer cell EMT and invasion through targeting Snail

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Abstract. – OBJECTIVE: Snail is an important factor in regulating epithelial mesenchymal transition (EMT). Its elevation is related to the enhancement of lung cancer invasion. MicroRNA-22 (MiR-22) plays a role in regulating lung cancer cell invasion. Bioinformatics analysis showed the complementary binding site between miR-22 and Snail. This study aimed to investigate the role of miR-22 in regulating Snail and affecting lung cancer cell invasion and metastasis.

MATERIALS AND METHODS: Dual luciferase assay confirmed the targeted relationship between miR-22 and Snail. MiR-22 and Snail expressions were compared in MRC-5, Anip973, and AGZY83-a cells. Cell colony formation and invasion were tested in Anip973 and AGYZ83-a cells. Anip973 and AGYZ83-a cells were treated by 5 ng/ml transforming growth factor β1 (TGF-β1) to detect miR-22, Snail, E-cadherin, and N-cadherin expressions. Anip973 cells were cultured in vitro and divided into five groups, including miR-Normal control (miR-NC), miR-22 mimic, small interfering RNA-Normal control (si-NC), si-Snail, and miR-22 mimic + si-Snail groups.

RESULTS: MiR-22 targeted inhibited Snail expression. MiR-22 significantly down-regulated, while Snail obviously elevated in Anip973 and AGYZ83-a cells compared with that in MRC-5 cells. Anip973 exhibited markedly stronger invasive and colony formation abilities than AGYZ83-a. TGFβ1 apparently reduced miR-22 and E-cadherin, whereas increased Snail and N-cadherin stronger in Anip973 than that in AGYZ83-a. MiR-22 mimic and/or si-Snail transfection significantly reduced Snail and N-cadherin levels, up-regulated E-cadherin expression, and attenuated cell colony formation and invasion.

CONCLUSIONS: Down-regulation of miR-22 plays a role in facilitating lung cancer cell EMT and invasion by elevating Snail. MiR-22 over-expression attenuated lung cancer cell EMT and invasion via targeted inhibiting Snail.

Key Words:
miR-22, Snail, EMT, Lung cancer, Invasion, Colony formation.

Introduction

Lung cancer is the most common malignant tumor with most rapid increase in morbidity and mortality. It accounts for the first in male and second in female morbidity and mortality among malignant tumors[1]. Limited by insidious tumorigenesis, most patients are in advanced stage when diagnosed, resulting in poor curative effect. Lung cancer is featured as high malignancy, rapid progression, easy to distant metastasis, high recurrence rate, and poor survival and prognosis[2,3]. Epithelial mesenchymal transition (EMT) is closely associated to tumor invasion, metastasis, and postoperative recurrence[4-6]. Snail promotes EMT to enhance cancer invasion by down-regulating E-cadherin to reduce cell adhesion and increase N-cadherin[7,8]. Snail abnormal over-expression is closely related to multiple cancers' EMT, invasion, and metastasis[9-12]. Several studies showed that Snail up-regulation participates in promoting lung cancer invasion, metastasis, and progression[13]. It is correlated with poor prognosis[14]. MiRNA is a type of endogenous single stranded non-coding RNA at the length of 21-24 nt discovered from eukaryote. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR. MiRNA expression and function in tu-

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morigenesis receive more and more attention[15,16]. Multiple studies[17-19] revealed that miR-22 plays a tumor suppressor role in various cancers. MiR-22 was found significantly declined in lung cancer, suggesting its potential carcinostasis role[20]. Bioinformatics analysis showed the complementary binding site between miR-22 and Snail. This study aimed to investigate the role of miR-22 in regulating Snail and affecting lung cancer cell invasion and metastasis.

Materials and Methods

Main Reagents and Instruments

Human high metastatic lung cancer cell line Anip973, low metastatic lung cancer cell line AGZY83-a, and normal pulmonary cell line MRC-5 were purchased from Jining Cell Culture Center (Shanghai, China). RPMI-1640, penicillin, and streptomycin were bought from Hyclone (South Logan, UT, USA). Opti-minimal essential medium (Opti-MEM) and fetal bovine serum (FBS) were got from Gibco (Grand Island, NY, USA). Lipofectamine 2000 was derived from Invitrogen Life Technologies (Carlsbad, CA, USA). EasyPure RNA Kit and Real-time PCR reagent TransScript Green One-Step qRT-PCR SuperMix were obtained from TransGen Biotech (Beijing, China). MiR-NC and miR-22 mimic were bought from Ribobio (Guangzhou, China). Mouse anti human Snail, E-cadherin, N-cadherin, and β-actin primary antibodies were got from Abcam Biotechnology (Cambridge, MA, USA). Bicinchoninic acid (BCA) protein quantification kit was purchased from Yeason (Shanghai, China). Transwell chamber was bought from Greiner Bio-One (Frickenhausen, Germany). Matrigel was derived from BD Biosciences (San Jose, CA, USA). Dual luciferase activity detection kit and pMIR luciferase gene reporter plasmid were purchased from Promega (Madison, WI, USA). TGF-β1 was obtained from Peprotech Co. Ltd. (Rocky Hill, NJ, USA).

Cell Culture and EMT Induction

Anip973 and AGZY83-a cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were passaged at 1:4. For EMT induction, Anip973 and AGZY83-a cells were seeded in six-well plate at 3×10^4/well for 24 h. Then cells were treated by 5 ng/ml TGF-β1 for 48 h to induce EMT.

Dual-Luciferase Reporter Gene Assay

The PCR products containing the full length of Snail gene 3'-UTR (forward, 5′-AATATATAA-ATTAACTGCTTTATTG-3′, reverse, 5′-CCCTC-GAGGCTCCCTCTTCCTCTCC-3′) segment were cloned to pMIR. Next, it was transformed to DH5α competent cells and sequenced to select the plasmid with correct sequence. Then pMIR-Snail-3'-UTR-wt (or pMIR-Snail-3'-UTR-mut) was co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-22 mimic (or miR-NC). The luciferase activity was detected according to the Dual-Glo® Luciferase Assay manual after it was cultured for 48 h.

Cell Transfection

Anip973 cells were cultured *in vitro* and divided into five groups, including miR-Normal control (miR-NC), miR-22 mimic, small interfering RNA-Normal control (si-NC), si-Snail, and miR-22 mimic + si-Snail groups. Nucleotide fragments and Lipofectamine 2000 were added to Opti-MEM and incubated at room temperature for 30 min, respectively. Then, they were added to the cells cultured in Opti-MEM. After 6 h incubation, the medium was changed back to RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were used for detection after 72 h.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using SPLIT RNA Extraction Kit and adopted for PCR reaction by TransScript Green One-Step qRT-PCR SuperMix. The reaction system contained 1 μg RNA template, 0.3 μM primers, 10 μl 2× TransStart Tip Green qPCR SuperMix, 0.4 μl RT Enzyme Mix, 0.4 μl Dye II, and ddH_2O. The reverse transcription condition was 37°C for 15 min and 98°C for 5 min. The PCR reaction was composed of 45°C reverse transcription for 5 min, 94°C pre-denaturation for 30 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Real-time PCR was performed on Bio-Rad CFX96/CFX connect to test the relative expression.

Western Blot Assay

The total protein was extracted by sodium dodecyl sulphate (SDS) buffer from cells. A total of 40 μg protein was separated by 8% to 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the membrane. Next, the membrane was blocked and incubated with primary antibody

at 4°C overnight (Snail, E-cadherin, N-cadherin, and β-actin at 1:1000, 1:2000, 1:2000, and 1:5000, respectively). Then, the membrane was incubated with secondary antibody (1:10000) for 60 min after washed by phosphate buffer solution-Tween-20 (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Colony Formation Assay

The cells were seeded in 10 cm dish at 100/well and cultured at 37°C and 5% CO_2 for 14 to 21 days. The dish was washed by PBS for twice and fixed by 4% paraformaldehyde when the macroscopic clone appeared. Next, the dish was stained by Giemsa for 20 min to count the clone with more than 10 cells. At last, the colony formation rate was calculated by (clone number/seeded cell number) ×100%.

Transwell Assay

A total of 600 µl RPMI-1640 medium containing 10% FBS were added to the 24-well plate. Then, transwell chamber paved matrigel was put onto the plate and added with cells resuspended in 200 µl serum-free RPMI-1640 medium (1×10^6/ml). After 48 h, the membrane was fixed in 4% paraformaldehyde for 30 min and stained by 0.1% crystal violet for 30 min. At last, the membrane was observed under the microscope.

Statistical Analysis

All data analyses were performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test. $p<0.05$ was considered as statistical significance.

Results

MiR-22 Targeted Regulated Snail Expression

Bioinformatics analysis showed the targeted binding site between miR-22 and 3'-UTR of Snail mRNA (Figure 1A). Dual luciferase assay revealed that miR-22 mimics transfection significantly declined the relative luciferase activity of HEK293 cells transfected by pMIR-Snail-3'-UTR-wt, while it exhibited no statistical impact on the luciferase activity in HEK293 cells transfected by pMIR-Snail-3'-UTR-mut

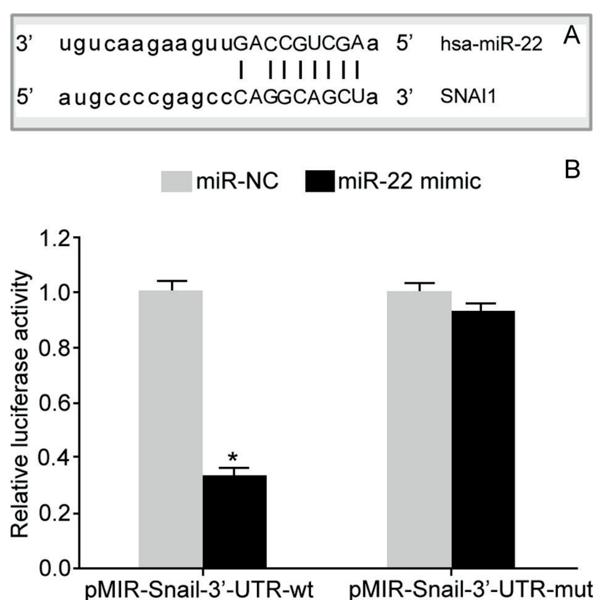

Figure 1. MiR-22 targeted regulated Snail expression. ***(A)*** The binding site between miR-22 the 3'-UTR of Snail mRNA; ***(B)*** Dual luciferase assay. $*p<0.05$, compared with miR-NC.

(Figure 1B), indicating the regulatory relationship between miR-22 and Snail mRNA.

MiR-22 Down-Regulated, While Snail Over-Expressed in Lung Cancer Cells

qRT-PCR showed that miR-22 was significantly lower, while Snail mRNA expression was significantly higher in Anip973 cells compared with MRC-5 cells. (Figure 2A). Western blot assay revealed that Snail protein level was highest in Anip973 cells, followed by AGZY83-a cells and MRC-5 cells (Figure 2B).

Snail Increased, While miR-22 Reduced in EMT Process of Lung Cancer Cells

Colony formation assay demonstrated that Anip973 cells exhibited markedly stronger clone formation ability than AGZY83-a cells (Figure 3A). Transwell assay exhibited that Anip973 cell invasive ability was significantly higher than AGZY83-a (Figure 3B). qRT-PCR showed that miR-22 obviously declined in Anip973 and AGZY83-a cells treated by TGF-β1, while it was more significant in Anip973 cells with stronger metastatic ability (Figure 3C). Western blot revealed that Snail and N-cadherin apparently up-regulated, while E-cadherin significantly decreased in lung cancer EMT process induced by TGF-β1 (Figure 3D).

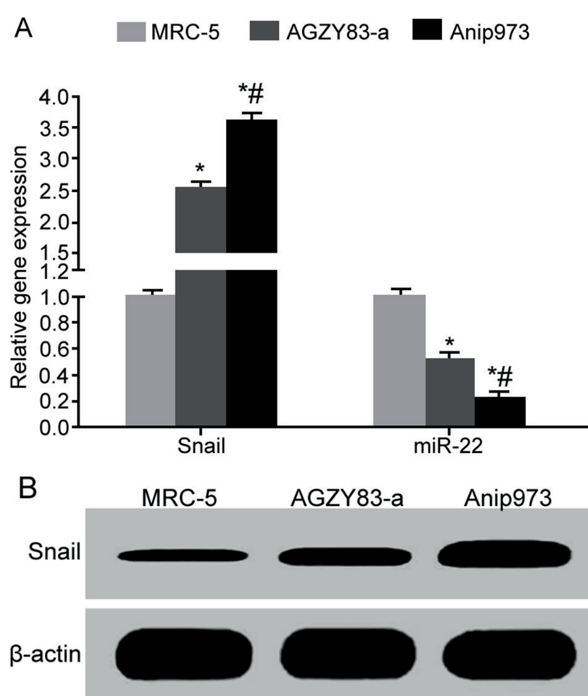

Figure 2. MiR-22 down-regulated, while Snail over-expressed in lung cancer cells. ***(A)*** qRT-PCR detection of gene expression. ***(B)*** Western blot detection of protein expression. $^{*}p<0.05$, compared with MRC-5 cells. $^{\#}p<0.05$, compared with AGZY83-a cells.

MiR-22 Over-Expression Suppressed EMT Process, Alleviated Clone Formation, and Attenuated Cell Invasion in Lung Cancer Cells

MiR-22 mimic and/or si-Snail transfection markedly reduced Snail and N-cadherin levels, up-regulated E-cadherin expression (Figure 4A), and attenuated cell colony formation (Figure 4B) and invasion (Figure 4C).

Discussion

Tumor invasion and metastasis are the comprehensive result of a series of biological processes, involving regulation of various factors, tumor cell migration and athletic ability, cell detach from the primary lesion, degradation of extracellular matrix, and migration through the blood or lymph system to form metastatic lesions[21,22]. EMT refers to the process of epithelial cells transforming into mesenchymal cells. In EMT process, epithelial cell polarity reduced or disappeared, while adhesion between cell and basement membrane declined, leading to extracellular matrix (ECM) degradation and migration and movement abilities enhancement. EMT is the initial step of cancer cells to obtain movement capacity and appear invasion and metastasis, which is closely related to surrounding tissue invasion, distant metastasis, postoperative recurrence, and poor prognosis[4-6]. In addition to tumor metastasis, EMT imbalance is also associated to abnormal embryonic development, pathological structure remodeling, and organ fibrosis. E-cadherin distributes in the joint between cells, thus playing an important role in maintaining epithelial cell polarity and cell-cell adhesion[23]. Different from E-cadherin, N-cadherin can induce EMT and enhance cell movement and migration abilities[24]. Snail is a kind of transcription regulatory factor containing zinc finger structure. It can bind to the E-box region of E-cadherin gene promoter to down-regulate E-cadherin expression in EMT process, thus reducing cell adhesion and enhancing cell migration, movement, invasion, and ECM degradation[7]. Snail abnormal upregulation is closely related to multiple cancers' EMT, invasion, and metastasis, such as prostate cancer[12], colorectal cancer[9], hepatic cancer[11], and bladder cancer[10]. Multiple studies reported that Snail involves in promoting lung cancer invasion, metastasis, and progression[13]; also, it is correlated with poor prognosis[14]. Ling et al[20] found that miR-22 significantly reduced in lung cancer, suggesting its potential carcinostasis role in lung cancer. Bioinformatics analysis showed the complementary binding site between miR-22 and Snail. This study aimed to investigate the role of miR-22 in regulating Snail and affecting lung cancer cell invasion and metastasis. Bioinformatics analysis showed the targeted binding site between miR-22 and 3'-UTR of Snail mRNA. Dual luciferase assay revealed that miR-22 mimics transfection significantly declined the relative luciferase activity of HEK293 cells transfected by pMIR-Snail-3'-UTR-wt, while it exhibited no statistical impact on the luciferase activity in HEK293 cells transfected by pMIR-Snail-3'-UTR-mut, indicating the regulatory relationship between miR-22 and Snail mRNA. MiR-22 was significantly lower, while Snail mRNA expression was obviously higher in Anip973 and AGZY83-a cells compared with MRC-5 cells. Snail level was higher, whereas miR-22 expression was lower in cells with stronger metastatic potential. It suggested that mIR-22 reduction induced Snail elevation not only participated in lung cancer tu-

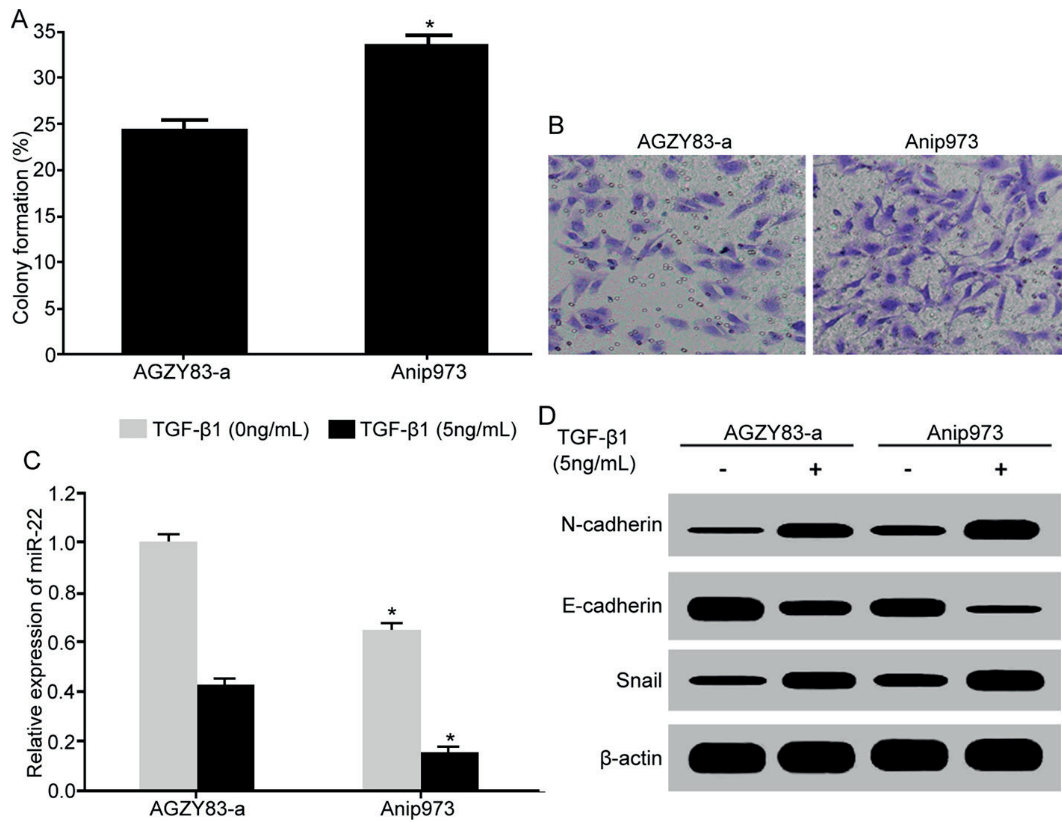

Figure 3. Snail increased, while miR-22 reduced in the EMT process of lung cancer cells. ***(A)*** Colony formation assay detection of clone formation. ***(B)*** Transwell assay detection of cell invasion. ***(C)*** qRT-PCR detection of miR-22 expression; ***(D)*** Western blot detection of protein expression. $^{*}p<0.05$, compared with AGZY83-a cells.

morigenesis, but also was involved in lung cancer metastasis potential. Xin et al[17] reported that miR-22 significantly down-regulated in A549 cells compared with human embryo lung fibroblasts, and it was obviously lower in tumor tissue compared with adjacent normal control. Shin et al[25] exhibited that miR-22 expression in pleural effusion from lung cancer patients was markedly lower than that from benign lesions, indicating that mIR-22 decrease may be related to lung cancer occurrence. Mairinger et al[26] found that miR-22 apparently reduced in lung cancer tissue and was correlated with pathological grading. Ling et al[20] demonstrated that miR-22 significantly

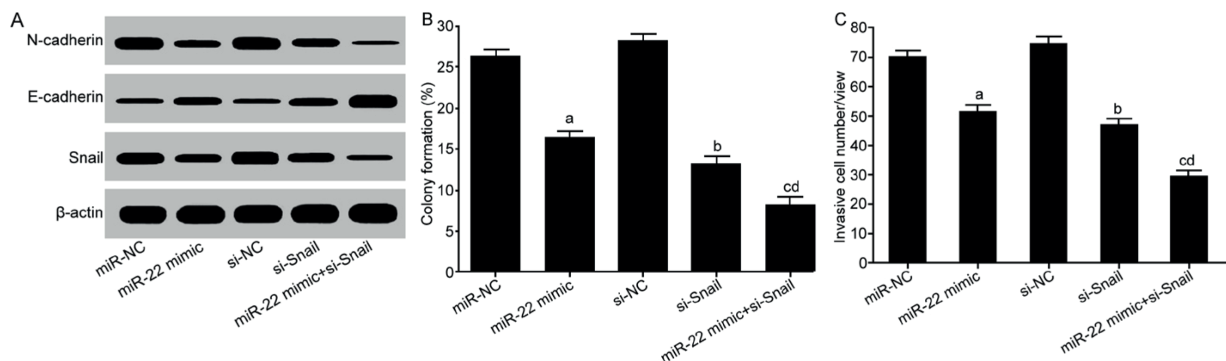

Figure 4. MiR-22 over-expression suppressed EMT process, alleviated clone formation, and attenuated cell invasion in lung cancer cells. ***(A)*** Western blot detection of protein expression; ***(B)*** Colony formation assay detection of clone formation. ***(C)*** Transwell assay detection of cell invasion. $^{a}p<0.05$, compared with miR-NC. $^{b}p<0.05$, compared with si-NC. $^{c}p<0.05$, compared with miR-22 mimic. $^{d}p<0.05$, compared with si-Snail group.

declined in lung cancer tissue compared with para-carcinoma tissue. Deng et al[27] showed that miR-22 level in lung cancer tissue was obviously lower than that in adjacent tissue. In this study, miR-22 expression in lung cancer cells was markedly higher than normal pulmonary cells, revealing that miR-22 participated in lung cancer tumorigenesis, which was similar with Xin et al[17], Mairinger et al[26], and Ling et al[20] findings. TGF-β1 induction significantly up-regulated Snail and N-cadherin expression, while decreased E-cadherin and miR-22 levels in Anip973 and AGZY83-a cells. However, it exhibited more obvious impact on Anip973 cells with stronger metastatic ability. Feng et al[8] discovered that Snail markedly increased in A549 EMT process induced by TGF-β1, which was similar with our results. Cai et al[28] demonstrated that miR-22 significantly declined in colorectal cancer HT-29 cell EMT induced by TGF-β. Su et al[29] revealed that miR-22 reduced in hepatic cancer cell EMT induced by folate deficiency. This study found that miR-22 apparently decreased in lung cancer EMT induced by TGF-β1, suggesting that miR-22 was a negative regulator of EMT, which was in accordance with Cai et al[28] and Su et al[29] results. Therefore, we selected Anip973 to further investigate the regulatory function of miR-22 and Snail in lung cancer cell EMT and invasion. MiR-22 mimic and/or si-Snail transfection markedly reduced Snail and N-cadherin levels, up-regulated E-cadherin expression, and attenuated cell colony formation and invasion. Xin et al[17] showed that miR-22 over-expression inhibited A549 cell proliferation, promoted cell apoptosis, and attenuated cell invasion by suppressing ACLY expression. Also, miR-22 suppressed A549 cell growth *in vivo* to weaken its oncogenicity by restraining ACLY[17]. Ling et al[20] revealed that miR-22 up-regulation inhibited lung cancer A549 and H1299 cell proliferation and alleviated cell invasion *in vitro* through targeting ErbB3. Moreover, miR-22 up-regulation apparently restrained A549 cell growth in nude mice[20]. Feng et al[8] reported that Snail siRNA significantly suppressed A549 cell EMT induced by TGF-β1. Yang et al[30] demonstrated that Snail down-regulation markedly blocked lung cancer EMT process, attenuated cell proliferation and invasion, and declined lung cancer cell growth and metastasis *in vivo*. In this work, miR-22 reduction and Snail over-expression are the enhancing and promoting factors of lung cancer EMT and invasion, which was in accordance with Ling et al[20] and Yang et al[30] findings. This study revealed that miR-22 down-regulation elevated Snail expression and promoted EMT process, thus playing a role in enhancing lung cancer cell colony formation and invasion.

Conclusions

Down-regulation of miR-22 plays a role in facilitating lung cancer cell EMT and invasion by elevating Snail. MiR-22 over-expression attenuated lung cancer cell EMT and invasion via targeted inhibiting Snail.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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