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Original Article

Long non-coding RNA ANRIL promotes proliferation, clonogenicity, invasion and migration of laryngeal squamous cell carcinoma by regulating miR-181a/Snai2 axis

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ABSTRACT

Background: Laryngeal squamous cell carcinoma (LSCC) is the common cancer with poor prognosis. Long non-coding RNA (lncRNA) ANRIL has been proven to play an important role in many cancers. However up to now, the role of ANRIL in LSCC is still poorly understood. The present study aimed to investigate the role and underlying mechanisms of ANRIL and miR-181a in LSCC.

Methods: Expression of ANRIL, miR-181a and Snai2 in both LSCC tissues and cells was determined by qRT-PCR. CCK-8 assay, colony formation assay, flow cytometry analysis and transwell assay were conducted to detect cell proliferation, clonogenicity, apoptosis, invasion and migration, respectively. The binding between ANRIL and miR-181a, as well miR-181a and Snai2 was confirmed by dual luciferase reporter assay. Western blotting was used to determine the protein levels of Snail, Slug, E-cadherin, N-cadherin and Vimentin.

Results: ANRIL was up-regulated while miR-181a was down-regulated in LSCC tissues. ANRIL was negatively correlated with miR-181a and was positively correlated with Snai1 and Snai2. Dual luciferase reporter assay showed ANRIL could directly sponge miR-181a to counteract its suppression on Snai2, serving as a positive regulator of Snai2. Either knockdown of ANRIL or overexpression of miR-181a significantly inhibited the proliferation, clonogenicity, invasion, migration and epithelial mesenchymal transformation (EMT), as well as promoted the apoptosis of LSCC cells, and knockdown of miR-181a reversed the effects.

Conclusion: Inhibition of ANRIL could suppress cell proliferation, clonogenicity, invasion and migration, as well as enhance cell apoptosis of LSCC cells through regulation of miR-181a/Snai2 axis, indicating that ANRIL might be a promising therapeutic target during the treatment of LSCC.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is the eleventh most common cancer all over the world and accounts for approximately 85–90% of all the larynx malignant tumors [1,2]. Since most LSCC patients are found to be advanced stages when diagnosed and LSCC is easy for recurrence and metastasis, the prognosis of LSCC patients, especially for advanced stage patients, remains poor [3,4]. The therapeutic treatment of LSCC has changed a lot in the past decade, but the treatment effect is still not very satisfactory [5]. Therefore, exploring the molecular mechanism of LSCC and finding key molecular targets is of great significance for the treatment of LSCC.

LncRNAs play important roles in cancer development [6]. It was reported that lncRNAs could be also used as biomarkers for prognosis of LSCC patients [7]. Antisense noncoding RNA in the INK4 locus (ANRIL) has been proven to promote cancer tumorigenesis in many cancers such as lung cancer [8], bladder cancer [9] and gastric cancer [10]. It was reported ANRIL was up-regulated in lung cancer, and increased expression of ANRIL promoted lung cancer cell metastasis and correlated with poor prognosis [8]. Furthermore,

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ANRIL promoted the invasion and metastasis through TGF-β/Smad signaling pathway in thyroid cancer cells [11]. However, the role of ANRIL in LSCC is still poorly understood.

The interaction between lncRNAs and microRNAs (miRNAs) has been noticed in many diseases, especially in cancer development [12]. Among the miRNAs, miR-181a is considered to suppress cancer development including lung cancer [13], colorectal cancer [14], breast cancer [15], etc. Moreover, it was found miR-181a could inhibit the progression of human LSCC [16]. However, no study focused on relationship between ANRIL and miR-181a and mechanisms for how miR-181a influences LSCC are also unclear. We found that there was a binding site of miR-181a on ANRIL through bioinformatic analysis. Based on the above evidence, the interaction between ANRIL and miR-181a may play a key role in the tumorigenesis of LSCC.

In this study, we revealed the role of ANRIL and miR-181a in LSCC. We demonstrated for the first time that ANRIL was up-regulated while miR-181a was suppressed in LSCC tissues. Inhibition of ANRIL could suppress cell proliferation, clonogenicity, invasion and migration, as well as enhance cell apoptosis of LSCC cells through sponging miR-181a. Furthermore, knockdown of ANRIL could inhibit epithelial mesenchymal transformation (EMT) by regulating Snai2-mediated EMT via sponging miR-181a. This research reveals mechanisms for ANRIL and miR-181a in development of LSCC and may provide some new research targets for LSCC treatment.

2. Methods and materials

2.1. Tissue samples

The present study used 28 paired LSCC and the adjacent normal tissues were obtained from patients who received surgery at the Second Hospital of Jilin University. All tissues were confirmed histologically. After surgically resected from the patients, the samples were stored at −80 °C. The study was approved by the Ethics Committee of the Second Hospital of Jilin University Hospital.

2.2. Cell culture

LSCC cell lines AMC-HN-8 and SNU-899 were purchased from ATCC (Manassas, VA, USA). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, CA, USA) containing 10% Gibco®fetal bovine serum (Gibco, Gaithersburg, MD, USA) at 37 °C and 5% CO_2.

2.3. Cell transfection

Cells were transfected with miR-181a mimics, miR-181a inhibitor, or the corresponding control mimics (mimics-NC or inhibitor-NC), as well as shANRIL, sh-NC, or pcDNA3.1-ANRIL and the blank vector (all purchased from GenePharma, Shanghai, China) after cultured to 50–70% confluence. Cell transfection was conducted with lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells transfected for 48 h were used for subsequent experiments.

2.4. CCK-8 assay

CCK-8 assay was used to determine the cell proliferation. Briefly, Cells were seeded at density of 3×10^5 in 96-well culture plates and were cultured overnight. After transfection for 48 h, 10 μL of CCK-8 solution was added to each well and cells were incubated at 37 °C for another 1 h. The absorbance of the solutions was detected at 490 nm by a SYNERGY-HT multiwell plate reader (Synergy HT, Winooski, VT, USA).

2.5. Colony formation assay

Briefly, cells were plated into 24-well dishes in 1 mL of medium. The cells were cultured for 2 week and then stained with crystal violet (Sigma–Aldrich, MO, USA) for 60 min. The positive colony formation was counted by quantity one software (Bio-Rad, Richmond, CA, USA).

2.6. Measurement of invasion and migration

Briefly, cells were plated in the top chamber with the non-coated membrane (BD Biosciences, CA, USA) in migration assay, and were plated in the top chamber with matrigel-coated membrane (#356234, pore size: 8.0 μm, BD Biosciences) in invasion assay. In both invasion and migration assays, cells were incubated for 24 h in serum-free media and were stained with 0.1% crystal violet, counted and photographed.

2.7. Dual luciferase reporter assay

We found that the binding sequence of miR-181a on ANRIL and 3′-untranslated region (3′-UTR) of Snai2 was 'GAAUGU' by Starbase (http://starbase.sysu.edu.cn/index.php) and Targetscan (http://www.targetscan.org/vert_71/)database, respectively. The binding sequence from 3′-UTR) of Snai2 or ANRIL containing the predicted miR-181abinding site was cloned into pmirGLO vector (Promega, Madison, WI, USA) to form the reporter vector Snai2-wild-type (WT-Snai2-3′UTR) or WT-ANRIL. To mutate the putative binding site of miR-181a in WT-Snai2-3′UTR or WT-ANRIL, site-mutations were performed to generate Snai2-mutated-type (MUT-Snai2-3′UTR) and MUT-ANRIL. Cells were co-transfected with wild-type/mut ANRIL or Snai2 3′-UTR, miR-181a mimics and miR-181a inhibitor using Lipofectamine 2000. The relative luciferase activity was normalized to values of Renilla luciferase activity using the dual-luciferase reporter assay (Promega, Madison, WI, USA).

2.8. Apoptotic cell analysis

For cell apoptosis analysis, after 48 h of transfection, cells were digested with trypsin and stained using Annexin V-FICT/PI double staining kit (Abcam, Cambridge, MA, USA). The cell apoptosis was measured by flow cytometry (BD Biosciences, San Jose, CA, USA).

2.9. RNA extraction and qRT-PCR

Total RNA was extracted from the tissues or LSCC cells using Trizol reagent (Tiangen Biotech, Beijing, China). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) was then used for converting RNA. qPCR reactions were performed using SYBR Green Master Mix (SolarbioScience& Technology Co., Ltd., Beijing, China) in an Exicycler™ 96 (Bioneer Corporation, Daejeon, Korea). The specific primers used in real-time PCR were listed as follows: ANRILforward 5′-TGCTCTATCCGCCAATCAGG-3′, reverse 5′-GGGCCTCAGTGGCACATACC-3'; hsa-miR-181a forward 5′ GCAACATTCAACGCTGTCG-3′, reverse 5′- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC-3'; Snai1 forward 5′-GAGCCCAGGCAGCTATTTCA-3′, reverse 5′-CATCGGTCAGACCAGAGCAC-3'; Snai2 forward 5′-GAGCATACAGCCCCATCACT-3′, reverse 5′- GGGTCTGAAAGCTTGGACTG-3'; U6 forward primer 5′-CTCGCTTCGGCAGCACA-3′, reverse primer 5′-AACGCTTCACGAATTTGCGT-3'; GAPDH forward, 5′-CCAGGTGGTCTCCTCTGA-3′ and reverse 5′-GCTGTAGCCAAATCGTTGT-3'. The relative RNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method. U6 and GAPDH were used as internal controls.

2.10. Western blotting

The protein levels of Snail, Slug, E-cadherin, N-cadherin and Vimentin were measured by Western blotting. Proteins were extracted, and the protein amount was quantified and then subjected to 10% SDS-PAGE. Samples were then transferred to PVDF membranes, blocked for 1 h using 5% not-fat milk and the membranes were then incubated with corresponding primary antibodies (Abcam, Cambridge, MA, USA) at 4 °C overnight. Primary antibodies used in this study were listed below: anti-Snail (ab53519, 1/1000), anti-Slug (ab180714, 1/1000), anti-E-cadherin (ab15148, 1/500), anti-N-cadherin (ab1820, 1/500), anti-Vimentin (ab137321, 1/1000) and GAPDH (ab181602, 1/1000). Then, membranes were incubated with corresponding secondary antibodies (Abcam) at 37 °C for 1 h. Films were scanned using the Pierce ECL Western blotting substrate (Pierce, Shanghai, China).

2.11. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) with Tukey's post-hoc test were used for analysis of two groups other there or more groups, respectively. Spearman's analysis was conducted to determine the correlation among ANRIL, miR-181a and Snai2 in LSCC tissues. $P < 0.05$ was considered as statistical different. All calculations were performed using SPSS 22.0.

3. Results

3.1. ANRIL was up-regulated while miR-181a was suppressed in LSCC tissues

As shown in Fig. 1A–C, compared with non-tumor tissue, the levels of both ANRIL and Snai2 were significantly up-regulated, while miR-181a was significantly down-regulated in LSCC tissues. Moreover, by using Spearman's analysis, we observed significant negative correlation between ANRIL and miR-181a, as well as between miR-181a and Snai2, while ANRIL and Snai2 showed remarkable positive correlation in LSCC tumor tissues (Fig. 1D–F), suggesting that ANRIL was up-regulated while miR-181a was down-regulated in LSCC tissues, and interactions might exist among the three factors. Furthermore, the results of spearman's analysis showed no significant correlation between ANRIL, miR-181a and Snai2 expression in normal tissues (Figure S1A-C).The expression of E-cadherin significantly decreased, while the expression of Snail (encoded by Snai1), Slug (encoded by Snai2), N-cadherin and Vimentin was significantly enhanced in LSCC tissues (Fig. 1G and H).

3.2. ANRIL promoted Snai2 expression via sponging miR-181a

To further investigate the interaction among ANRIL, miR-181a, Snai1 and Snai2, we successfully knocked down or overexpressed ANRIL in both AMC-HN-8 and SNU-899 cells by

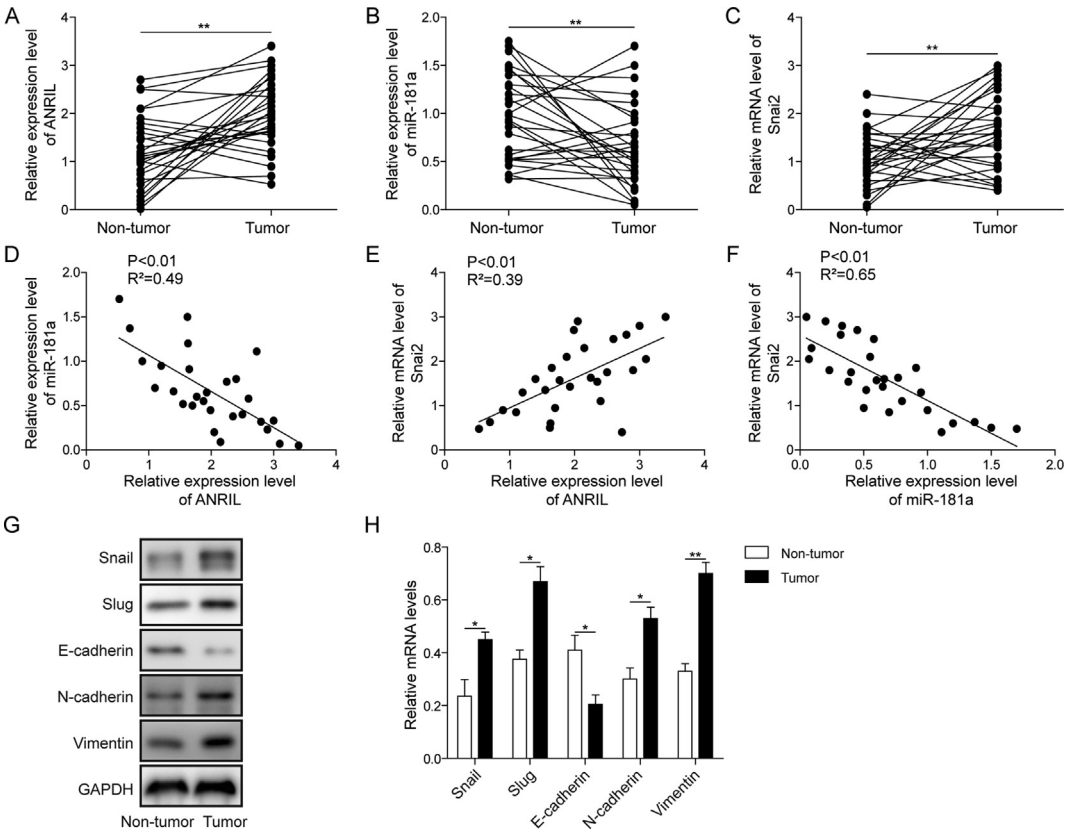

Fig. 1. ANRIL was up-regulated while miR-181a was decreased in LSCC tissues. The levels of ANRIL (A), miR-181a (B) and Snai2 (C) in LSCC tissues were determined by qRT-PCR. The correlation between ANRIL and miR-181a (D), ANRIL and Snai2 (E), and miR-181a and Snai2 (F) was determined by Spearman's analysis. (G) The EMT-related protein expression level in LSCC tissues was determined by Western boltting. (H)Quantitative analysis of Western blotting. The levels of their expression were normalized to GAPDH. *$p < 0.05$ and **$p < 0.01$.

shANRIL or pcDNA3.1-ANRIL. Results showed when ANRIL was inhibited, the levels of miR-181a were apparently enhanced while the levels of both Snai1and Snai2 were remarkably suppressed (Fig. 2A). However, overexpression of ANRIL led to opposite results (Fig. 2A). Interestingly, when miR-181a was overexpressed, levels of Snai1 and Snai2 were significantly inhibited, while inhibition of miR-181a led to opposite results (Fig. 2B). These results further indicated that ANRIL positively regulated miR-181a and negatively regulates Snai1 and Snai2 in LSCC cells. Additionally, after transfection of miR-181a mimics and inhibitor, we detected the expression level of ANRIL and the results showed the expression of ANRIL had no significant change compared with the NC group (Figure S1D and E). The predicted biding modes among ANRIL, miR-181a, Snai2 were shown in Fig. 2C and D by bioinformatics analysis. Furthermore, Starbase and Targetscan database analysis revealed that there was no miR-181a binding site on Snai1. Results of dual luciferase reporter assay showed the relative luciferase activity was significantly lower in ANRIL-WT when miR-181a was overexpressed, while the relative luciferase activity was significantly higher in ANRIL-WT when miR-181a was inhibited (Fig. 2E). However, no significant difference was found in ANRIL-MUT. Similar results were also observed in Snai2-WT and Snai2-MUT (Fig. 2F). All these results indicated ANRIL could up-regulate Snai2 by sponging miR-181a in LSCC cells.

3.3. Knockdown of ANRIL regulated cell proliferation, clonogenicity and apoptosis of LSCC cells through up-regulating miR-181a

As shown in Fig. 3A–C, the proliferation and clonogenicity were remarkably suppressed when ANRIL was inhibited or miR-181a was overexpressed. Besides, when ANRIL was overexpressed, the cell proliferation and clonogenicity were remarkably enhanced (Fig. 3A–C). However, when cells were co-transfected with shANRIL and miR-181a inhibitor, the decreased cell proliferation and clonogenicity by shANRIL was reversed (Fig. 3A–C). On the contrary, the cell apoptosis was significantly promoted in both AMC-HN-8 and SNU-899 cells when ANRIL was knocked down or miR-181a was overexpressed, and inhibiting miR-181a significantly reversed the increasing apoptosis by shANRIL (Fig. 3D and E). The overexpression of ANRIL reduced the cell apoptosis rate in AMC-HN-8 cells (Fig. 3D and E). All these results suggested knockdown of ANRIL could suppress cell proliferation and clonogenicity, and enhance cell apoptosis of LSCC cells through up-regulating miR-181a.

3.4. Knockdown of ANRIL suppressed the invasion and migration of LSCC cells via regulating miR-181a

It was observed in LSCC cells, the transfection with shANRIL or miR-181a mimics suppressed both cell invasion and migration of LSCC cells (Fig. 4A–D). However, when cells were co-transfected

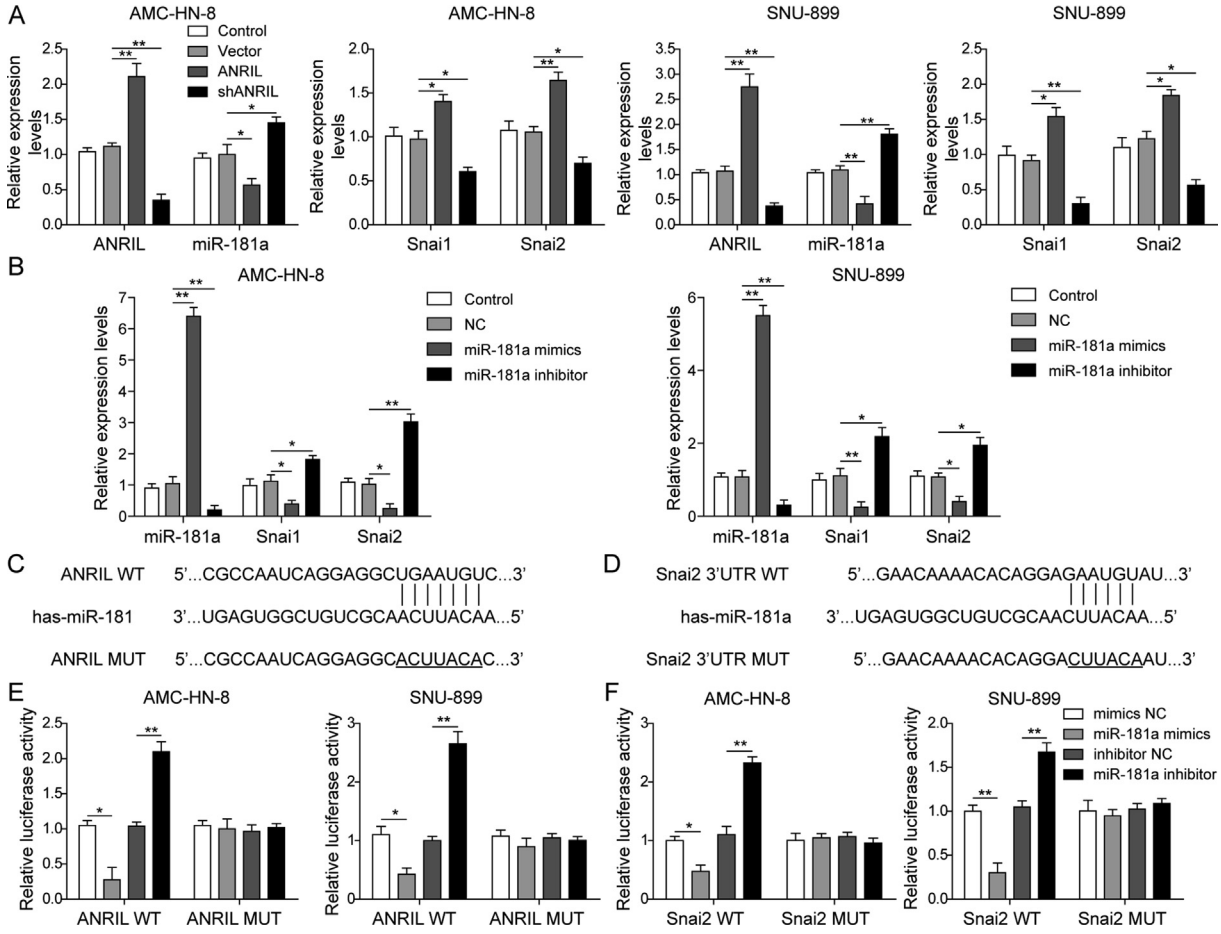

Fig. 2. ANRIL promoted the expression of Snai2 via sponging miR-181a. (A) The levels of ANRIL, miR-181a, Snai1 and Snai2 were determined by qRT-PCR in AMC-HN-8 and SNU-899 cells transfected with shANRIL or pcDNA3.1-ANRIL. (B) The levels of miR-181a, Snai1 and Snai2 were determined by qRT-PCR in LSCC cells transfected with miR-181a mimics, inhibitor or NC. (C) Sequence alignment between ANRIL and miR-181a. (D) Sequence alignment between miR-181a and the 3′-UTR of Snai2. (E) The relative luciferase activity of ANRIL-WT and ANRIL-MUT was measured by dual luciferase assay. (F) The relative luciferase activity of Snai2-WT and Snai2-MUT was measured by dual luciferase assay. $^{*}p < 0.05$ and $^{**}p < 0.01$.

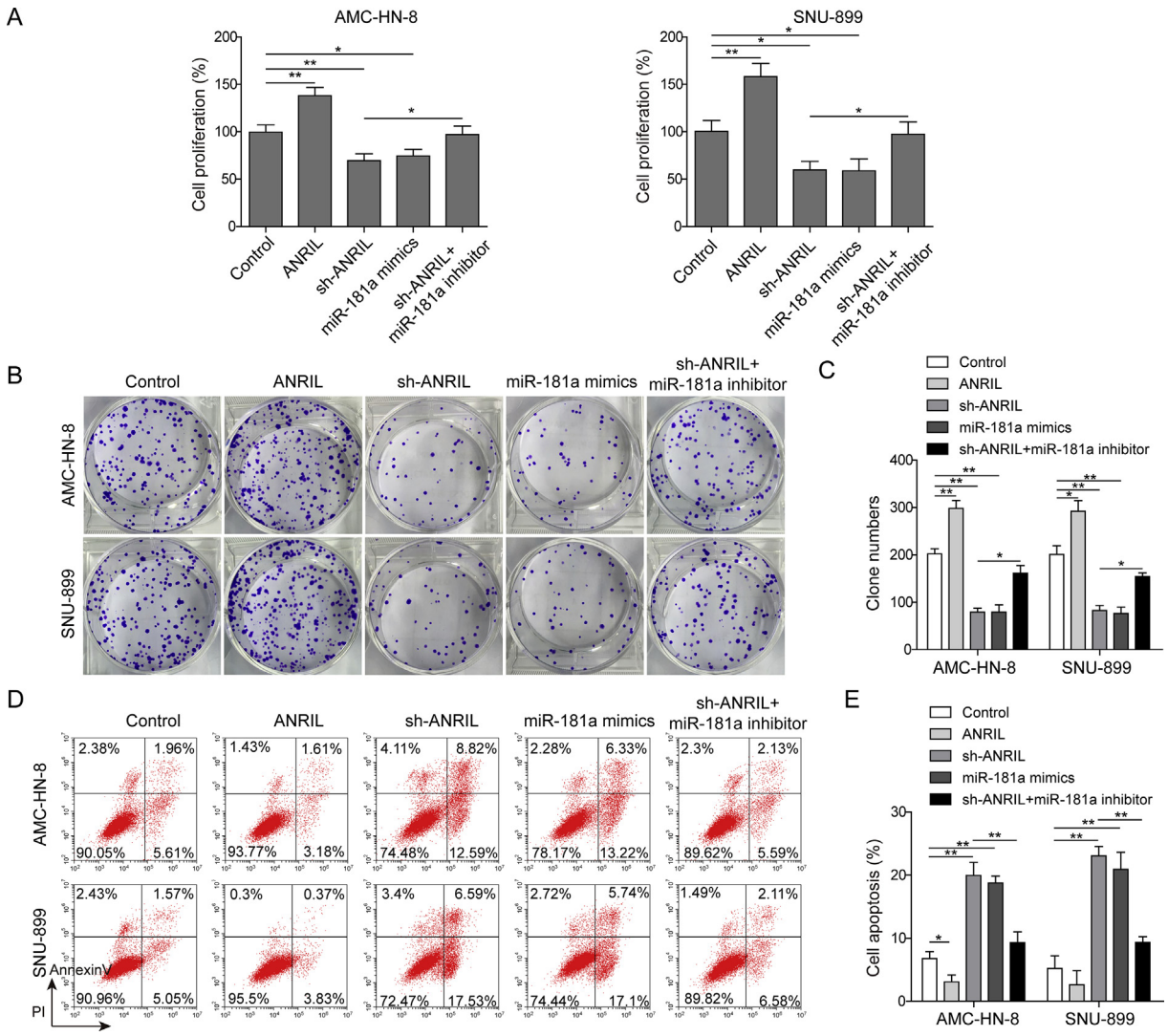

Fig. 3. Knockdown of ANRIL inhibited the cell proliferation and clonogenicity, and promoted the apoptosis of LSCC cells by increasing miR-181a. The cell proliferation was evaluated by CCK-8 assay (A). The cell clonogenicity was determined by colony formation assay (B) and the clone numbers (C) were counted in both AMC-HN-8 and SNU-899 cells transfected with shANRIL, pcDNA3.1-ANRIL, miR-181a mimics or miR-181a inhibitor. The cell apoptosis was determined by the flow cytometry analysis (D) and the apoptosis rate (E) was calculated for different groups of cells. $^{*}p < 0.05$ and $^{**}p < 0.01$.

with both shANRIL and miR-181a inhibitor, the decreased cell invasion and migration by shANRIL were significantly reversed by inhibition of miR-181a. Meanwhile, overexpression of ANRIL promoted cell invasion and migration of LSCC cells (Fig. 4A–D). These results demonstrated that knockdown of ANRIL could inhibit the invasion and migration through elevating miR-181a.

3.5. Knockdown of ANRIL inhibited the EMT of LSCC cells by increasing miR-181a

In both AMC-HN-8 and SNU-899 cells, protein levels of Snail, Slug, N-cadherin and Vimentin were all significantly down-regulated and protein level of E-cadherin was remarkably up-regulated when ANRIL was knocked down or miR-181a was over-expressed (Fig. 5A and B). However, when co-transfected with both shANRIL and miR-181a inhibitor, the above effects were significantly reversed. Furthermore, overexpression of ANRIL increased the expression of Snail, Slug, N-cadherin and Vimentin, and decreased the expression level of E-cadherin (Fig. 5A and B). These results indicated that inhibition of ANRIL could suppress the EMT of LSCC cells, and the process might be through up-regulation miR-181a.

4. Discussion

LSCC is one of the most common cancers with high mortality and poor prognosis. Though there are many studies on treatment and basic research of LSCC, the underlying mechanisms for LSCC development are unclear. In recent decades, the roles of lncRNAs in cancer development are more and more noticed [17]. Studies reported that ANRIL could promote cancer development in many cancers. However, up to now, no study focused on the role of ANRIL in LSCC. In the present research, we reported for the first time that ANRIL could promote proliferation, clonogenicity, invasion and migration of LSCC by regulating miR-181a/Snai2 axis and suppressing EMT. This research might reveal the role of ANRIL and miR-181a in LSCC and contribute to LSCC treatment.

ANRIL is a lncRNA which contains 19 exons, spans a region of 126 kb, and is transcribed in a 3834 bp mRNA in the antisense orientation of the p15/CDKN2B-p16/CDKN2Ap14/ARF gene cluster

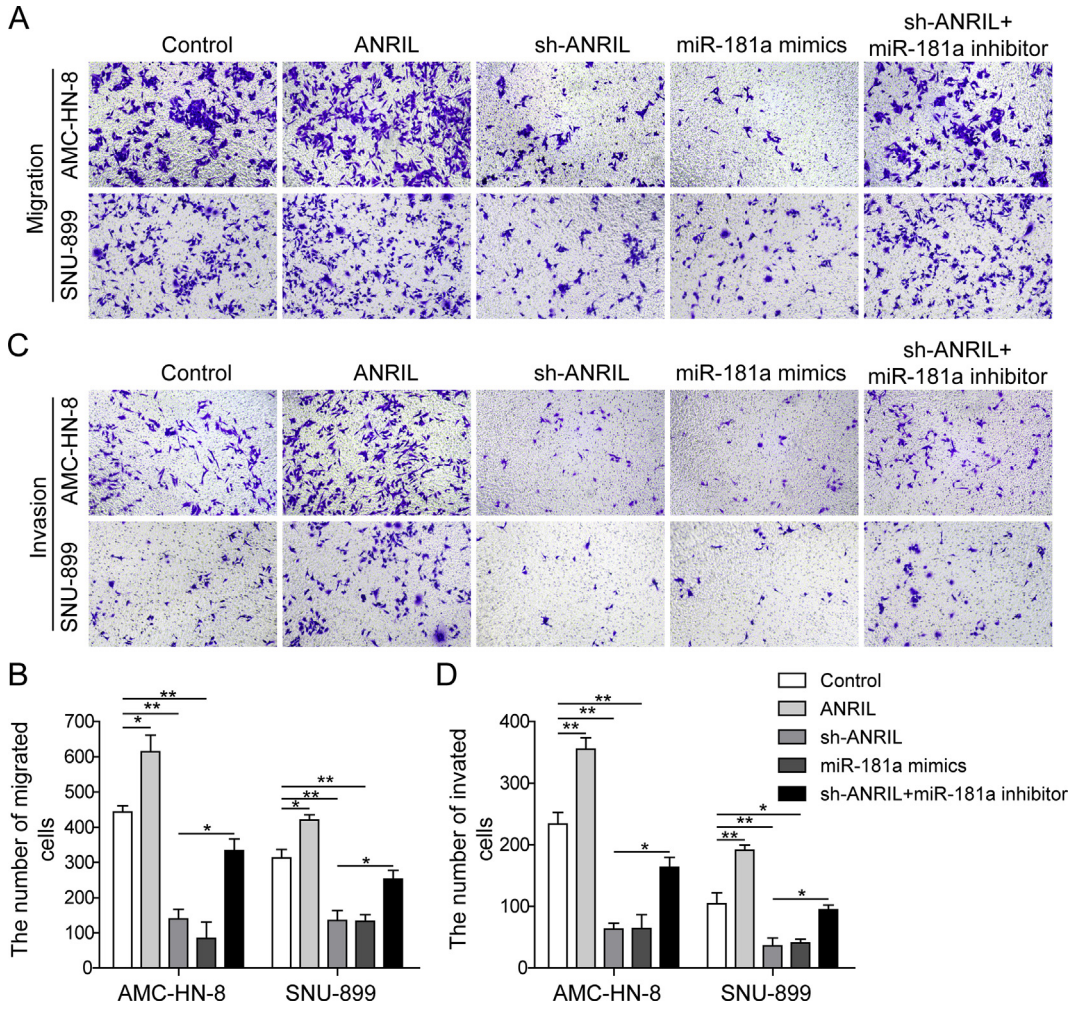

Fig. 4. Knockdown of ANRIL inhibited the invasion and migration of LSCC cells via up-regulating miR-181a. The cell migration (A) was determined by transwell assay and the migrated cells were calculated (B) in both AMC-HN-8 and SNU-899 cells transfected with shANRIL, pcDNA3.1-ANRIL, miR-181a mimics or miR-181a inhibitor. The cell invasion (C) was also measured by transwell assay and the invaded cells were calculated (D) in both AMC-HN-8 and SNU-899 cells transfected with shANRIL, miR-181a mimics or miR-181a inhibitor. *p < 0.05 and **p < 0.01.

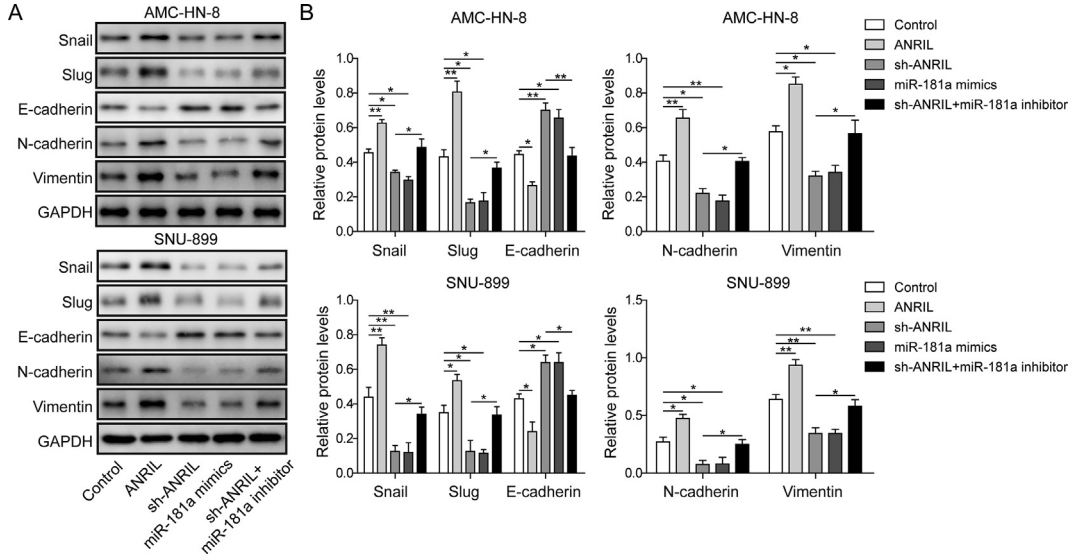

Fig. 5. Knockdown of ANRIL inhibited the EMT of LSCC cells via targeting miR-181a. The protein levels of Snail, Slug, N-cadherin, Vimentin and E-cadherin were determined by Western blotting (A) and the quantitative results (B) in both AMC-HN-8 and SNU-899 cells transfected with shANRIL, pcDNA3.1-ANRIL, miR-181a mimics or miR-181a inhibitor.*p < 0.05 and **p < 0.01.

[18]. Studies showed ANRIL played important roles in many diseases including atherosclerosis [19] and coronary heart disease [20]. The role of ANRIL as a tumor promoter has also been reported in several researches. Zhang et al., showed ANRIL was significantly up-regulated in cervical cancer, and ANRIL could promote proliferation and invasive ability through regulation of miR-186 [21]. Nie et al., demonstrated ANRIL could promote cell proliferation and inhibit cell apoptosis of non-small cell lung cancer cells [22]. It was also found that overexpression of ANRIL could promote EMT in pancreatic cancer [23]. In this paper, we also found ANRIL was up-regulated in LSCC tissues, and inhibition of ANRIL could inhibit cell proliferation, clonogenicity, invasion, migration and EMT, as well as enhance the cell apoptosis of LSCC cells.

miR-181a, one of the miRNAs, is one of the most well-studied miRNAs which belongs to miR-181 family. miR-181a has been demonstrated to be involved in diverse cellular functions such as growth, proliferation, death, survival, and maintenance [5]. Studies also showed miR-181a suppressed cancer development in many cancers, including LSCC. Zhao et al., demonstrated that miR-181a was down-regulated in LSCC, and overexpression of miR-181a could suppress migration and promote apoptosis of LSCC by targeting GATA6 [24]. It was also shown miR-181a could inhibit cell migration and angiogenesis of breast and colon cancer cells by down-regulating MMP-14 [25]. Here, we also showed miR-181a was decreased in LSCC, and miR-181a could inhibit the cancer development. The interaction between lncRNAs and miRNAs has been noticed and studied in recent years [26]. A recent study reported that miRNAs and lncRNAs have a crosstalk in cancers metastasis and this interaction may be through the regulation of EMT [12]. Several studies also reported the interaction between miR-181a and lncRNAs. Chang et al., demonstrated lncRNA-XIST could promote hepatocellular carcinoma by sponging miR-181a [27]. It was also found lncRNAs such as CRNDE [28], CASC2 [29] and NEAT1 [30] could target miR-181a in cancer development. However, to our best of knowledge, no study reported correlation between miR-181a and ANRIL. In our research, we reported that ANRIL could competitively bind to miR-181a in LSCC, and the interaction between ANRIL and miR-181 also influenced EMT process.

Snail (encoded by Snai1) and Slug (encoded by Snai2) belong to the Snail superfamily of zing-finger transcription factors, which are key factors in EMT [31]. Zhang et al., found the up-regulation of Snai2 could enhance the invasion ability of breast cancer cells [32]. Baygi et al., observed Snai2 was significant up-regulated in prostate carcinoma cell lines and could promote the EMT [33]. It was also showed the miR-613 could down-regulate Snai2 expression and further suppress EMT in LSCC [34]. In the present study, we also found Snai2 was increased in LSCC. He et al., demonstrated Snai2 was a direct target of miR-181a in salivary adenoid cystic carcinoma [35]. In this research, we also demonstrated Snai2 was a direct target of miR-181a in LSCC. However, up to now no study reported relationship between ANRIL and Snai2.

In conclusion, we investigated the role of ANRIL and miR-181a in LSCC. Results showed ANRIL was up-regulated while miR-181a was decreased in LSCC tissues. Inhibition of ANRIL could suppress cell proliferation, clonogenicity, invasion, migration and EMT, as well as enhance cell apoptosis of LSCC cells through regulation of miR-181a/Snai2 axis. This research may give deeper insights for ANRIL and miR-181a in development of LSCC and provide some new potential targets for cancer treatment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.07.007.

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