RUNX3 inhibits laryngeal squamous cell carcinoma malignancy under the regulation of miR-148a-3p/DNMT1 axis

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Laryngeal squamous cell carcinoma (LSCC) is a highly aggressive malignant cancer and accounts for 1% to 2% of all malignancies diagnosed worldwide. Runt-related transcription factor 3 (RUNX3), an important tumor suppressor, is known to related lymph node metastasis and the development of LSCC. However, the biological roles and potential mechanisms RUNX3 expression was not well understood. In this study, we reported that the RUNX3 was significantly downregulated and highly methylated in LSCC compared with their matched normal. The enforced expression of RUNX3 inhibited LSCC cell migration, invasion, and proliferation, whereas the inhibition of RUNX3 did the opposite. We identified that RUNX3 was regulated by miR-148a-3p and found that the expression level of miR-148a-3p was significantly decreased and positively related with the expression of RUNX3 in LSCC. We also identified that DNA methyltransferase enzyme DNA (cytosine-5')-methyltransferase 1 (DNMT1) was targeted by miR-148a-3p in LSCC. The knockdown of DNMT1 promoted the expression of RUNX3 and inhibited migration, invasion, and proliferation in LSCC cells. In summary, our study demonstrated that miR-148a-3p may regulate RUNX3 expression through the modulation of DNMT1-dependent DNA methylation in LSCC, providing a novel target and a potential therapeutic pathway against LSCC.

KEYWORDS
DNMT1, LSCC, methylation, miR-148a-3p, RUNX3

1 INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignancies in the head and neck region, and it is the 11th common cancer worldwide.1 Despite encouraging progress in the diagnosis and treatment, including surgical resection, radiotherapy, and chemotherapy, the overall survival rate of LSCC remains unfavorable for the past 10 years.2 A recent study has shown that the overall 1- and 2-year survival rates of LSCC patients without treatment are only 56.4% and 26.5%, respectively.3 There is very little information about the precise molecular pathways involved in LSCC development and procession. Therefore, a better understanding of the molecular mechanisms of LSCC progression and a new strategy for LSCC treatment are urgently needed.

Three members of the runt-related transcription factor (RUNX) family genes, RUNX1, RUNX2, and RUNX3 transcription factors, are known as important developmental regulators in the inception and progression of a variety of human cancers.4 RUNX3 is a tumor suppressor gene that participates in the regulation of cell proliferation and apoptosis. It has been reported that the changes in RUNX3 protein levels or functions are associated with various epithelial cancers.
including LSCC. RUNX3 plays a tumor suppressor role in most related cancers. A recent study reported that the hypermethylation of the RUNX3 promoter region downregulates its expression and modulates the progression of carcinoma.

The methylation of DNA is established and maintained by DNA (cytosine-5-)methyltransferases (DNMTs). There are 3 enzymatically active mammalian DNMTs, namely, DNMT1, DNMT3 alpha (DNMT3A), and DNMT3 beta (DNMT3B). The most abundant one is DNMT1, which is primarily responsible for the maintenance and regulation of DNA methylation patterns. Previous studies have shown that the significantly increased the expression of DNMT1 protein positively correlates with the hypermethylation status of CpG islands of several gene in epithelial cancer, including RUNX3, SSTR2, and CYB.

MicroRNAs (miRNAs), a group of short, noncoding RNAs of 18 to 25 nucleotides, were originally identified in the early 1990s in Caenorhabditis elegans. MiRNAs negatively regulate gene expression at the posttranscriptional level via imperfect hybridization to 3' untranslated region (3'-UTR) in their target messenger RNA (mRNA). Functionally, miRNAs play important roles in various biological processes, including cell development, differentiation, proliferation, apoptosis, fat metabolism, and stem cell renewal. Evidence for the involvement of miRNAs in cancer came from the observation that miRNAs were dysregulated in various tumor tissues. In recent years, an increasing number of studies have reported the influence of miRNA on numerous cancer-related processes such as proliferation, migration, and invasion. There have also been studies illustrated that several miRNAs may contribute to carcinogenesis and cancer progression in LSCC. A previous report demonstrated that miR-148a-3p was significantly downregulated in LSCC. Furthermore, it has been verified that DNMT1 is a target of miR-148a-3p. Therefore, we hypothesized that miR-148a-3p could regulate RUNX3 gene expression through the modulation of DNMT1-dependent DNA methylation in LSCC.

Our present study found a function for RUNX3 in LSCC. We first identified that the expression of RUNX3 is affected by miRNA-induced promoter methylation regulation. We confirmed the biological role of RUNX3 in LSCC and investigated the associations of RUNX3 expression and RUNX3 promoter methylation with miR-148a expression in LSCC. In addition, miR-148a-3p mimic or demethylation regent was transfected into LSCC to detect the effect of miR-148a-3p and demethylation on RUNX3 expression and LSCC progression.

2 MATERIALS AND METHODS

2.1 Tissue sample

A total 12 laryngeal carcinoma and 12 tumor-adjacent normal samples were used in this study. All samples were harvested at The First Affiliated Hospital of Henan University of Science and Technology from September 2013 to October 2015. The tumor-adjacent normal tissues were obtained from surgically resected tissues that were located more than 5 cm away from the tumors. All tissue samples were immediately snap-frozen in liquid nitrogen and stored in liquid at ~80°C until further processing. The study was approved by the Ethics Committee of Henan University of Science and Technology and informed consent was obtained.

2.2 Cell lines

The human laryngeal cancer cell lines, Tu212 (in-CL-0228) and Tu686 (in-CL-0228), were purchased from Jiningshiye Company (Shanghai, China). Control cell line 293T (ATCC® CRL-3216™) was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured separately in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, California, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, Utah, USA), 100 U/mL penicillin, or 100 μg/mL streptomycin in a humified atmosphere at 37°C in 5% CO2.

2.3 RNA extraction and quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from laryngeal carcinoma and corresponding adjacent nonneoplastic tissues or LSCC and control cell lines using Trizol reagent (Invitrogen, Carlsbad, California, USA) and quantified using the NanoDrop 1000 (NanoDrop Technologies, Rockland, Delaware, USA). The RNA integrity was assessed using standard denaturing agarose gel electrophoresis. Reverse transcriptase polymerase chain reaction (qRT-PCR) was performed as described previously. The gene-specific primers were as follows: RUNX3 (forward: 5′-TC TGT AAG GCC CAA AG TGG GTA-3′; reverse: 5′-ACC TCA GCA TG AC AAT ATG TCA CAA-3′), miR-148a-3p (forward: 5′-AGC AGT TC AGT AGT GCA CTA CAG-3′; reverse: 5′-GCA GTT GCC GAG GTT GGA-3′), DNMT1 (forward: 5′-CCA TCA GCA ATT CTA CCA-3′; reverse: 5′-CGT TC TC CTT GTC TTC TCT-3′), β-actin (forward: 5′-TTG GAC AGT CAG CGT CAT CTT-3′; reverse: 5′-ACA CCC AAT AGC ACC AAA TTC GTC-3′), and U6 (forward: 5′-AGA GCC TGT GGT GTC CG-3′; reverse: 5′-CAT CTT CAA AGC ACT TCC CT-3′). β-Actin and U6 SnRNA were used as the internal control for mRNA and miRNA, respectively. Relative gene expression was quantified by the 2-ΔΔCt method. All PCR reactions were performed on a StepOne Plus RT-PCR instrument (Applied Biosystems, Shanghai, China).

2.4 Sodium bisulfite modification and MS-PCR

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Qiagen, Valencia, California, USA). The bisulfite conversion was performed using an EpTect Bisulfite (Qiagen) in accordance with the product manual. The methylation status of RUNX3 was analyzed by MSP as described previously. MSP assays were repeated at least twice to verify the reproducibility of the assay.

2.5 Protein extraction and western blot

Total protein was extracted using a Qproteome Mammalian Protein Prep Kit (Qiagen) in accordance with the manufacturer’s instruction. Typically, 20 μg of the protein was loaded per lane. Protein samples were resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature and incubated at 4°C overnight with human specific
anti-RUNX3 antibody (1:500, sc-376591; Santa Cruz, CA, USA) and anti-DNMT1 antibody(1:500, sc-271729; Santa Cruz). Sequentially, the secondary antibodies were conjugated to horseradish peroxidase, and the proteins were visualized via chemiluminescence (Beyotime, China). β-Actin (1:200, ACTB; sc-47778; Santa Cruz, CA, USA) was used to normalize the quantity of the protein.

2.6 Generating overexpression and transfection

Phoenix-Ampho cells were transfected with an empty retroviral vector (MSCVpac) or a retroviral vector containing the human RUNX3 complementary DNA (MSCVpac-hRUNX3) by the calcium phosphate method as previously described. A medium collected from the transfected Phoenix-Ampho cells was used to infect Tu686 cells. Positively infected cells were selected with 1 μg/mL of puromycin to generate Tu686/Control and Tu686/RUNX3 cells. Using Lipofectamine™2000, the combinator plasmids and 100 nM Hsa-miR-148a-3p mimics, miR-148a-3p mimic control, Hsa-miR-148a-3p inhibitor, and inhibitor control (Ambion, Austin, Texas, USA) were transfected into Tu686 cells, respectively, for 48 hours.

2.7 Construction of the luciferase reporter vector

A 300-bp sequence from the 3′-UTR of RUNX3 containing a putative miR-148a-3p binding site was amplified by PCR using the cDNA of Tu686 cells as a template. The sequence for the mutation within the miR-148a-3p binding site was amplified by the point mutation method using the KOD-Plus mutagenesis Kit (TOYOBO, Osaka, Japan) according to the protocols provided. After cloning into the pGEM-T vector (Promega, Madison, Wisconsin, USA), the PCR product was purified and inserted into a pmIR-REPORT luciferase miRNA expression reporter vector (Ambion) using SpeI and HindIII (Takara Biotechnology, Dalian, China). The recombinant plasmids were confirmed by DNA sequencing. The firefly luciferase and the renilla luciferase activity were detected by a fluorescence detector (Promega). Relative luciferase activity was normalized to renilla luciferase activity for each transfected well. The experiment was replicated 3 times for data calculation.

2.8 Cell proliferation and colony formation assays

For cell proliferation assay, 2 × 10^2 transfected cells/well were plated into 96-well plates and cultured at the indicated time points (1, 3, 5, and 7 day). Then 10 μL CCK-8 reagent (DoJinDo, Japan) was added to each well. The OD450 nm value was determined by a microplate reader (Bio-Tek Instruments, Winooski, Vermont). For colony formation assay, 1 × 10^3 transfected cells/well were seeded in 6-well plate and cultured for 2 weeks. Then cells were fixed with 4% paraformaldehyde for 20 minutes and stained with 1% crystal violet. The total number of colonies was counted under a light microscope (Olympus, Tokyo, Japan).

2.9 Wound healing assay

The migration ability of cells was analyzed by a wound healing assay. One day before scratching, cells were seeded into 12-well plates to almost total confluence in 48 hours. A scratching wound was made by scraping the middle of the cell monolayer with a sterile micropipette tip. After all detached cells were washed away with phosphate-buffered saline, the cells were cultured with medium containing 10% FCS, images of the cells migrating into the wound area were captured at 48 hours by an inverted microscope (100×), and their distances were recorded.

2.10 Transwell assay

The invasive behaviors of indicated cells were analyzed by Transwell chamber (Corning Costar-Corp., Cambridge, Massachusetts) assay. Briefly, 2 × 10^5 transfected cells in serum-free medium were added to each upper chamber precoated with Matrigel matrix, and 500 μL of DMEM medium containing 10% FBS was added to the lower chamber to serve as chemoattractant. After 24 hours incubation, cells on the surface of upper chamber were removed by scraping with a cotton swab, and the invasive cells on the lower membrane surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, photographed, and quantified by counting them in 5 random fields by a light microscope (Olympus, Tokyo, Japan).

2.11 Statistical analysis

All the experiments in this study were performed 3 times. All data from this study were obtained from at least 3 independent experiments and were expressed as mean ± SD. All statistical analyses were conducted using the software of SPSS version 11.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Statistical analysis between 2 samples was performed using the Student t-test. The chi-square or the Fisher exact test was used to evaluate the differences in proportion. Difference were considered significant when P < .05.

3 RESULTS

3.1 RUNX3 is significantly downregulated and highly methylated in LSCC

To detected the expression of RUNX3 in LSCC, we examined the mRNA and protein expression level of RUNX3 in cancer tissues and 2 LSCC cell lines by qRT-PCR and western blot, respectively. As shown in Figure 1A and B, the mRNA and protein levels of RUNX3 were significantly downregulated in tumor samples compared with the corresponding normal tissues (P < .01). The results in LSCC cell lines, Tu212 and Tu686, were consistent with that in tumor samples (Figure 1D and E). The promoter methylation status of RUNX3 was measured by MSP in cancer and adjacent normal tissues. As shown in Figure 1G and I, RUNX3 showed obvious methylation in cancer tissues and LSCC cell lines, whereas almost no methylation in adjacent normal tissues or control cell line was found. The results illustrated that the expression and promoter methylation levels of RUNX3 were both significantly elevated in LSCC.
3.2 | RUNX3 overexpression inhibits proliferation, colony formation, migration, and invasion of Tu686 cells

To investigate the role of RUNX3 in LSCC tumorigenesis, we analyzed the functional effects of RUNX3 in Tu686 cells transfected with RUNX3 overexpression vector. qRT-PCR assay confirmed that RUNX3 expression level was significantly increased in Tu686 cells after transfection (Figure 2A and B). Then we investigated the effects of RUNX3 overexpression on cell proliferation and colony formation. Compared with control group, the overexpression of RUNX3 in Tu686 cells significantly inhibited cell proliferation (Figure 2C) and colony formation (Figure 2D and E). To further study the effect of RUNX3 on LSCC cells, migration and invasion assays were performed in Tu686 cells using wound healing and invasion chamber assay, respectively. It was found that RUNX3 overexpression substantially inhibited the migration and invasion capabilities of Tu686 cells (Figure 2F and G).

3.3 | MiR-148a-3p is downregulated and modulate the methylation of RUNX3 by target DNMT1 in LSCC

To examine the effects of miR-148a-3p on RUNX3 gene expression, we detected the expression of miR-148a-3p in LSCC tissue and cell lines using qRT-PCR. The results demonstrated that the expression of miR-148a-3p was significantly decreased in cancer tissues compared with adjacent normal tissue (Figure 3A) and inhibited in LSCC cell lines compared with control cells (Figure 3B). Furthermore, we observed a positive correlation between the mRNA expression level of RUNX3 and the miR-148a-3p expression level (Figure 3C). Previous studies have already confirmed that miR-148a-3p could modulate DNMT1 protein expression.12 We first observed a negative correlation between the mRNA expression level of DNMT1 and miR-148a-3p (Figure 3D). In addition, we predicted DNMT1 can be the target of miR-148a-3p using bioinformatic software, such as Target Scan, miRbase, RegRNA, and so on. According to the results, the potential binding target sites of miR-148a-3p were found in the 3′-UTR of DNMT1 gene (Figure 3E). The luciferase reported vectors of DNMT1-wt and DNMT1-mut, which contained the wild type and mutant type of potential binding sequence in 3′-UTR of DNMT1 gene (Figure 3E). The luciferase reporter vectors of DNMT1-wt and DNMT1-mut, which contained the wild type and mutant type of potential binding sequence in 3′-UTR of DNMT1 gene (Figure 3E). The luciferase reporter vectors of DNMT1-wt and DNMT1-mut, which contained the wild type and mutant type of potential binding sequence in 3′-UTR of DNMT1 gene (Figure 3E). The luciferase reporter vectors of DNMT1-wt and DNMT1-mut, which contained the wild type and mutant type of potential binding sequence in 3′-UTR of DNMT1 gene (Figure 3E). The luciferase reporter vectors of DNMT1-wt and DNMT1-mut, which contained the wild type and mutant type of potential binding sequence in 3′-UTR of DNMT1 gene (Figure 3E).
3.4 | Overexpression of miR-148a-3p promotes proliferation, migration, and invasion by upregulated RUNX3 gene expression

To further confirm that the effect of miR-148a-3p on Tu686 cells was dependent on the upregulation of RUNX3 expression, miR-148a-3p mimic and mimic control were transfected into Tu686 cells. qRT-PCR and western blot assay showed that miR-148a-3p overexpression could increase RUNX3 expression on mRNA level (Figure 4A) and protein level (Figure 4B and C) in Tu686 cells. Furthermore, the expression of DNMT1 were significantly decreased with miR-148a-3p mimic transfection in both mRNA and protein level. Then we analyzed the cell proliferation, migration, and invasion, respectively. The results demonstrated that miR-148a-3p overexpression significantly inhibited cell proliferation, migration, and invasion ability of Tu686 cells compared with mimic control group (Figure 4D–F). These data indicated that miR-148a-3p exerts tumor suppressive effects on LSCC cells partially through inhibiting DNMT1 expression and promoting RUNX3 expression.

3.5 | DNMT1 contributes to the aberrant methylation of RUNX3

To further study a DNMT1-dependent mechanism of RUNX3 alteration, we assayed the expression of RUNX3 after the knockdown of DNMT1 in Tu686 cells. In addition, 5-Aza-DdR, a common demethylation reagent, was used to inhibit the promoter methylation of RUNX3. The results showed that DNMT1 knockdown and 5-Aza-DdR treatment increased the protein expression of RUNX3 (Figure 5A). In addition, an decrease in promoter methylation level of RUNX3 was observed in DNMT1 siRNA and 5-Aza-DdR-treated cells (Figure 5B). Furthermore, we analyzed the cell proliferation, migration, and invasion in 5-Aza-DdR and DNMT1 siRNA groups. The results
**FIGURE 3** MiR-148a-3p expression and target reaction with DNMT1 in LSCC. A, The expression of miR-148a-3p in LSCC cancer and adjacent normal tissues were detected using qRT-PCR. *P < .05 compared with the normal group. B, The expression of miR-148a-3p in Tu212, Tu686, and 293T cells were detected using qRT-PCR. *P < .05, **P < .01 compared with the 293T group. C, Positive correlation between miR-148a levels and RUNX3 mRNA levels. D, Negative correlation between miR-148a levels and DNMT1 mRNA levels. E, Schematic view of miR-148a-3p putative targeting site in the wild type (wt) and mutant (mut) of DNMT1. F, Relative luciferase activity in Tu686 cells cotransfected with miR-148a-3p mimic or mimic control together with luciferase reporter vector constructs with the sequences of either wild type or mutant type of DNMT1 3′-UTR. *P < .05 compared with miR-148a-3p mimic + DNMT1 wt group. G, Relative luciferase activity in Tu686 cells cotransfected with miR-148a-3p inhibitor or inhibitor control together with luciferase reporter vector wt-DNMT1 or mut-DNMT1. *P < .05 compared with miR-148a-3p inhibitor + DNMT1 wt group. Luciferase activities were represented as firefly luciferase normalized to renilla luciferase. Data are represented as the mean ± SD of 3 experiments.

**FIGURE 4** Overexpression of miR-148a-3p regulates Tu686 cell development by promoting RUNX3 gene expression. Tu686 cells were transfected with miR-148a-3p mimic control or miR-148a-3p mimic for 48 hours, respectively. A, RUNX3 and DNMT1 mRNA expression were determined by qRT-PCR. (B and C) RUNX3 and ANMT1 protein expression were measured by western blot. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. Cell proliferation (E), migration (F), and invasion (G) were determined in Tu686 cells transfected with miR-148a-3p mimic control or miR-148a-3p mimic. Data are represented as the mean ± SD of 3 experiments. *P < .05, **P < .01 compared with the miR-148a-3p mimic control group.
demonstrated that 5-Aza-DdR and DNMT1 siRNA treatment in Tu686 cells could inhibit cell proliferation, migration, and invasion (Figure 5C, D, and E). Therefore, suggested that DNMT1 contributes to the aberrant methylation of RUNX3 and furthermore causes dysregulation of RUNX3 expression and modulates LSCC progression.

4 | DISCUSSION

LSCC is one of the most common cancers with high incidence and mortality. RUNX3 has been implicated as a tumor suppressor in various cancers, including LSCC. Studies have shown that RUNX3 is most prominent in the dorsal root ganglia, hematopoietic cells, and gastrointestinal tract, where it is thought to play a role in cell differentiation and development. Although present in many cell types, the role of RUNX3 in normal cellular development is not fully understood. Nowadays, epigenetic biomarkers based on the analysis of DNA methylation status have been proven to be useful in both detection and the prognostication of carcinoma. Thus, the current study was designed to investigate the potential role and underlying mechanism of RUNX3 and its promoter methylation in LSCC.

In the present study, we first found that RUNX3 expression was downregulated in LSCC tissues and cell lines compared with normal control, which was consistent with the results in other cancers such as gastric cancer, non-small cell lung cancer, esophageal cancer, and so on. To investigate the function of RUNX3 in LSCC, we detected the cell proliferation, migration, and invasion in LSCC cell line Tu686 transfected with RUNX3 overexpression vector. Obviously, our data showed that RUNX3 could inhibit tumor progression, including inhibiting cell proliferation, colony formation, cell migration, and invasion in vitro. These results suggested a tumor suppress role of RUNX3 in LSCC. It has been reported that promoter region of RUNX3 was hypermethylated in 56% of tumors. Our results indicate that the methylation level of RUNX3 was significantly increased in LSCC cancer tissue compared with adjacent normal tissue, hinted at apparent modification may be involved in tumor development. The previously mentioned results suggest that epigenetic modification participate in the regulating of the tumor occurrence and progression of LSCC. However, the underlying mechanism of aberrant RUNX3 promoter hypermethylation has not been fully elucidated.

The identification of the molecular mechanism responsible for the activation of RUNX3 gene expression may contribute to the field of LSCC therapy. Numerous miRNAs have been identified to play significant roles in the pathogenesis of LSCC through regulating cell proliferation, metastasis, invasion, and apoptosis. For example, Tian et al reported that miR-27a acts as an oncogene in LSCC through the downregulation of PLK2. Xu et al demonstrated that miR-106b could increase the proliferation and invasion of laryngeal carcinoma cells of
through targeting RUNX3, suggesting miR-106 acted as oncogene in LSCC. Here, we found that miR-148a-3p expression was downregulated in LSCC tissues compared with adjacent normal tissues, and miR-148a-3p overexpression inhibited LSCC cell proliferation, migration, and invasion in vitro. These will be helpful for the better understanding of LSCC carcinogenesis and may provide a new therapeutic target for LSCC treatment.

It has been reported that miRNA target and directly regulated the expression of RUNX3. For instance, miR-532-5p, which had a sequence complementary to the 3’ UTR of RUNX3, was highly expressed in melanoma and could suppress RUNX3 expression.20 Another study demonstrated that miR-138 regulate the balance of Th1/Th2 by directly suppress the expression of RUNX3 in psoriasis.21 These studies suggest that RUNX3 can be regulated by miRNAs and modulate the progression of not only cancers but also other diseases. However, in the present study, the expression of RUNX3 and miR-148a-3p was positively correlated in LSCC, illustrated that there are no target reaction between RUNX3 and miR-148a-3p, and suggested miR-148a-3p may regulated RUNX3 through another way. A previous study reported that miR-148a-3p modulates RUNX3 expression via altering the promoter methylation status in gastric cancer.22 Another study demonstrated that the IncRNA H19 promoted LSCC progression via miR-148a-3p and DNMT1.23 In this study, DNMT1 was predicted to be the target gene of miR-148a-3p using bioinformatic software programs. Furthermore, to verify the targeting reaction between miR-148a-3p and DNMT1, the luciferase reporter vectors of wild type and mutant DNMT1 were constructed. The results showed that the overexpression of miR-148a-3p inhibited luciferase activity when Tu686 cells were transfected with DNMT1-wt luciferase reporter system, but not in DNMT1-mut groups. Moreover, the inhibition of miR-148a-3p increased luciferase expression in DNMT1-wt transfection group compared with DNMT1-mut groups. These results demonstrated that DNMT1 is a direct target for miR-148a-3p, which was consistent with the former study.23

It has been reported that miRNAs played a critical role in the progression of various cancers through regulating different cellular progression.24 In our study, the results demonstrated that miR-148a-3p overexpression inhibited cell proliferation, migration, and invasion in LSCC. The directly target of miR-148a-3p DNMT1 is considered as the primary DNA methyltransferase enzyme in gene methylation. Study has reported that the promoter methylation of RUNX3 is frequently associated with DNMT1 expression.25 Our study demonstrated that miR-148a-3p overexpression downregulate DNMT expression, which decreased the methylation of RUNX3, and the RUNX3 protein levels was increased. Thus, we further investigate the role of promoter methylation of RUNX3 in LSCC. It has been accepted that the deoxycytidine analog 5-aza-dC is a DNA methylation inhibitor, which can covalent binding with DNMTs during the methylation reaction. Kang et al26 reported that RUNX3 was upregulated by treatment with the methylation inhibitor 5-aza-dC in colorectal cancer. In our study, Tu686 cells treated with 5-aza-dC or DNMT1 siRNA significantly decreased promoter methylation of RUNX3 and elevated RUNX3 mRNA and protein expression. This was consistent with the study of Kang et al. Furthermore, similar to the effect of miR-148a-3p overexpression, 5-aza-dC or DNMT1 siRNA inhibited cell proliferation, migration, and invasion. These results provide evidence for miRNA-induced regulation of promoter methylation through DNMTs and suggested that RUNX3 can be regulated by miR-148a-3p, possibly by the regulation of promoter methylation in LSCC.

In conclusion, the present study first showed that RUNX3 and miR-148a-3p was significantly decreased in LSCC, and RUNX3 or miR-148a-3p overexpression promotes LSCC cell proliferation, colony formation, migration, and invasion in vitro. We also proved that miR-148a-3p could regulate RUNX3 expression through the modulation of DNMT1-dependent DNA methylation in LSCC. These results suggested that RUNX3 functioned as a tumor suppressor in LSCC, which could be regulated by miR-148a-3p through targeting DNMT1, and that RUNX3 might serve as a potential target for LSCC treatment.

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