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LncRNATCF7 up-regulates DNMT1 mediated by HPV-16 E6 and regulates biological behavior of cervical cancer cells by inhibiting miR-155

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Abstract. – OBJECTIVE: This work aimed to study the mechanism of lncRNATCF7 upregulating DNMT1 mediated by HPV-16 E6 and regulating the biological behavior of cervical cancer cells by inhibiting miR-155.

PATIENTS AND METHODS: HPV-16 E6 enhanced DNMT1 expression in cervical cancer cells, which was detected by Western blotting. The expression of miR-155 in cervical cancer was detected by qPCR, the interaction between TCF-7 and miR-155 by Dual-Luciferase reporter gene. The changes in invasion ability of cervical cancer cells and the effect of miR-155 on the invasion ability of cervical cancer cells after inhibiting TCF-7 were detected by the transwell invasion assay, while changes in migration ability of cervical cancer cells and the effect of miR-155 on migration ability of cervical cancer cells after inhibiting TCF-7 were observed by the scratch assay. The effect of inhibiting TCF-7 on the tumor size and volume of cervical cancer was detected by the subcutaneous tumor formation in nude mice.

RESULTS: E6 expression was significantly inhibited by E6 siRNA. The knockdown of endogenous HPV-16 E6 markedly inhibited the expression of DNMT1; TCF-7 specifically bound to the 3' UTR of miR-155; inhibition of TCF-7 can inhibit invasion and migration of cervical cancer cells; enhanced miR-155 after the inhibition of TCF-7 can promote the invasion and migration of cervical cancer cells; compared with NC group, the tumor volume and weight of TCF-7-siRNA group tumor-bearing was significantly reduced.

CONCLUSIONS: TCF-7 plays an important role in the development of cervical cancer. TCF-7 can target miR-155 to regulate the invasion and migration of cervical cancer cells.

Key Words:
TCF-7, Cervical cancer, MiR-155, DNMT1, Transwell.

Introduction

Cervical cancer is the second most common cancer in female malignancies[1,2]. The current research indicates that the persistent infection with high-risk human papillomavirus (HR-HPV), especially HPV-16, is a key risk factor for cervical cancer[3]. HPV-16 infections were observed in more than 70% of cervical cancer cases[4]. HPV-16 E6 and E7 are two key oncoproteins that trigger a range of carcinogenic processes. E6 binds to the cellular protein ubiquitin-protein ligase E3A (UBE3A) and initiates the proteasomal degradation of p53, a well-known tumor suppressor gene, while E7 induces the degradation of pRb[5]. Lu et al[6] have reported that HPV-16 E6 is also associated with epigenetic regulation of disorders during cervical cancer. For example, E6 and E7 gene silencing[7] lead to a reduction in methylation of tumor suppressor genes in several human cervical cancer cell lines. Knocking out E6 in HPV-16-positive human cervical cancer SiHa and CaSki cells[8] directly lead to the inhibition of DNMT1 protein by decreasing the promoter activity. Indeed, DNMT1 is an important enzyme regulating the DNA methylation[9], and its abnormal regulation is related to the malignant phenotype and methylation gene expression in cervical cancer cells[10].

LncRNA TCF7 is a long-chain non-coding RNA that is abnormally expressed in cervical cancer[11]. Furthermore, it acts as an upstream regulator of miR-206 and promotes the development of gallbladder carcinoma[12] and regulates tumor radiosensitivity by interfering with miR-145 expression[13]. In the biological function, miR-155 overex-

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pression and low expression of TCF7 in cervical cancer cells have similar effects in inhibiting cell proliferation and invasion[14]. MiR-155 has previously been shown to be a tumor suppressor and is usually down-regulated in cervical cancer[15]. Hypermethylation of the promoter region has been reported to be responsible for the down-regulation of miR-155 in breast cancer[16] and esophageal cancer[17]. However, it is unclear whether miR-155 is down-regulated in cervical cancer and whether there is any association between them.

In this study, a significant difference in the expression of TCF7 in cervical cancer cells was demonstrated, which was mediated by HPV-16 E6-enhanced DNMT1 up-regulation. We also observed a mutual regulation between TCF7 and miR-155, which is related to the migration and invasion behavior of cervical cancer cells.

Patients and Methods

Tissue Acquisition and Cell Culture

After the informed consent of patients undergoing cervical cancer surgery in the Chengwu Hospital Affiliated to Taishan Medical College, cervical cancer and adjacent normal tissue samples were obtained. Tumors and corresponding non-tumor fresh specimens were snap frozen in liquid nitrogen and stored at –80°C immediately after excision to extract RNA and protein. This investigation was approved by the Research Ethics Committee of Chengwu Hospital Affiliated to Taishan Medical College. Human cervical cancer cell lines SiHa and CaSki cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. This study was approved by the ethics committee of Chengwu People's Hospital.

Cell Transfection and qRT-PCR

SiHa and CaSki cells used 100 nM HPV-16 E6 siRNA, 100 nM TCF7 siRNA. The TRIzol kit was used in qRT-PCR to extract the total RNA from cervical cancer patients and control tissues. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit to synthesize the cDNA sense strand. The RT-PCR reaction was then performed in a Real Time PCR system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was considered an internal reference. The reaction conditions were pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 60°C for 32 s, and the dissolution curve was detected after 50 cycles. After the test was completed, the Ct value of each sample was automatically analyzed by a computer system and the RNA relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated three times. The lncRNA TCF7 primer sequence were as follows: 5'-GTGGAGGATCGGATTTTAGCAAACT-3' (forward) and 5'-CCTATGGGATCGGGCAGTGCAAAGT-3' (reverse). MiR-155 primer sequences: 5'-TTGAGCGGAGTCGGTAGGGCAAATCG-3' (forward) and 5'-GCCTATCTTTTAGGGCGAGCA-3' (reverse).

Western Blot Analysis

Total protein from cells was extracted by using radioimmunoprecipitation assay (RIPA) buffer. Total protein concentration was measured by the bicinchoninic acid (BCA) protein assay. Samples containing 30 μg of protein were loaded into each lane, separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane for routine Western blot analysis. The antibodies used included Anti-E6, anti DNMT1. The membrane was washed and embedded with the corresponding horseradish peroxidase (HRP)-labeled secondary antibody. Protein signals were detected using chemiluminescent substrates and the intensity of each band was quantified by the ImageJ software (NIH, Bethesda, MD, USA). The experiment was repeated three times.

Dual-Luciferase Assay

The TCF7 fragment containing the predicted wild-type (WT) or mutant (MT) miR-155 binding site was chemically synthesized, and the Dual-Luciferase miRNA targeted downstream of the luciferase gene of the expression vector. The recombinant plasmids were designated pmirGLO-miR-155-WT, pmirGLO-miR-155-MUT, respectively, and the cells were cultured in 12-well plates, and then Lipofectamine 2000 and recombinant reporter plasmid were used, and cells were harvested and lysed 24 h later. The luciferase activity was determined using a Dual-Luciferase assay reporter system according to the manufacturer's instructions. The experiment was repeated three times.

Scratch Healing Experiment

The si-TCF-7 and NC group cells were cultured to the log phase of growth. A cell-free area was created by scratching a single layer of cells in a standard manner using a 200 μl sterile pipette tip.

The medium was aspirated and replaced with fresh complete medium, and then the cells were incubated at 37°C for 24 h. Cell migration after 24 h was recorded and photographed. The migration residual gap between the migrating keratinocytes was measured at five random intervals under each experimental condition to compare the percentage of the original scratch width as comparison data.

Transwell Invasion Experiment

1×10^5 cells were suspended in 200 μL of serum-free RPMI-1640 medium and then inoculated into the upper chamber. To create a chemoattractant environment in the lower chamber, RPMI-1640 supplemented with 20% FBS was filled in. After incubation for 24 h in a cell culture incubator, the cells on the top surface of the insert were removed. The cells on the bottom surface were fixed with 4% polyoxymethylene, and the number of invading cells was counted after staining with 0.1% crystal violet. The experiment was repeated three times.

Nude Mouse Tumor Xenograft Model

Cervical cancer cells of 1×10^6 concentration in the si-TCF-7 group and NC group were injected into the armpits of 4-6-week-old nude mice, respectively. The volume and mass growth of nude mouse tumor xenografts were examined after 8 weeks of feeding. The allograft size was measured according to the following formula: volume = 1/2 (shortest diameter) 2 × (longest diameter).

Statistical Analysis

We used Spearman's Rank for correlation analysis. For the remaining experiments, statistical analysis was performed using the Student's *t*-test. Data were expressed as mean ± standard deviation and the significance level was $p<0.05$, which was considered statistically significant.

Results

HPV-16 E6 Enhances the Expression of DNMT1 in Cervical Cancer Cells

The expressions of HPV-16 E6 and DNMT1 in HPV-16-positive SiHa and CaSki cells were first investigated. E6 expression was significantly inhibited by E6 siRNA and the knockdown of endogenous HPV-16 E6 significantly inhibited DNMT1 expression (Figure 1).

HPV-16 E6 and DNMT1 Knockdown Increased the Expression of MiR-155 in Cervical Cancer

Cervical cancer cells were transfected with DNMT1 siRNA, qRT-PCR was performed to detect the miRNA expression of miR-155 after transfected with HPV-16 E6 siRNA [(1.05±0.14) *vs.* (1.12±0.18) *vs.* (5.86±0.36), $p<0.05$], the results showed that the expression level of miR-155 can be significantly increased (Figure 2).

Dual-Luciferase Assay to Detect the Relationship Between TCF7 and MiR-155 in Cervical Cancer Cells

Based on our bioinformatics analysis, we found that TCF7 had a putative binding site to miR-155 (Figure 3A). The Dual-Luciferase assay showed that TCF7-siRNA significantly inhibited the luciferase activity of miR-155 (Figure 3B). The results indicated that TCF7-siRNA can specifically bind to the 3'UTR of miR-155 and can regulate its expression activity and level.

TCF7 Inhibits Migration and Invasion of Cervical Cancer Cells by Down-Regulating MiR-155

Results of the scratch healing experiment showed (Figure 4B) that the cell migration distance of the TCF7-siRNA group was sig-

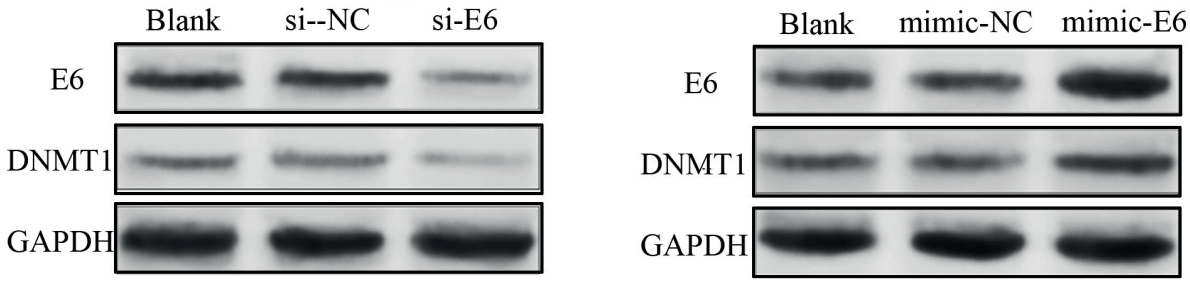

Figure 1. Western blotting detection of DNMT1 expression in cervical cancer cells.

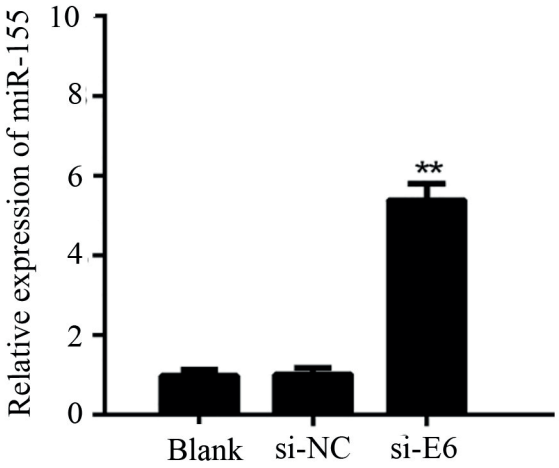

Figure 2. Expression of miR-155 in cervical cancer cells.

nificantly lower than that of the NC control group [(83.52±8.59) um *vs.* (23.48±5.28) um, $p<0.05$]. The difference between the two groups was statistically significant. The results of the transwell invasion showed that the number of cells in the TCF7-siRNA group through Matrigel was 42.54±8.12, which was significantly less than that in the NC group (123.52±15.24). The difference was statistically significant ($p<0.05$). It was indicated that inhibition of TCF7 expression can enhance the invasive ability of cervical cancer cells. It was shown that the inhibition of the expression of TCF7 inhibited the migration of cervical cancer cells.

Reversal Effect of MiR-155 on Migration and Invasion of Cervical Cancer Cells

The results of the scratch healing experiment showed (Figure 5A) that the cell migration distance of the TCF7-siRNA+miR-155-mimic group was significantly higher than that of the TCF73-siRNA group [(83.52±8.59) *vs.* (36.45±6.15) um, $p<0.05$], the difference between the two groups was statistically significant, indicating that overexpression of miR-155 can enhance the migration ability of cervical cancer cells and enhance the effect of TCF-7. The results of transwell invasion assay showed (Figure 5B) that the number of cells in the TCF7-siRNA+miR-155-mimic group through Matrigel was 79.65±7.61, which was significantly less than that in the TCF7-siRNA group (221.14±21.35), the difference was statistically significant ($p<0.05$). This indicated that overexpression of miR-155 can reverse the effect of TCF7 on the invasive ability of cervical cancer

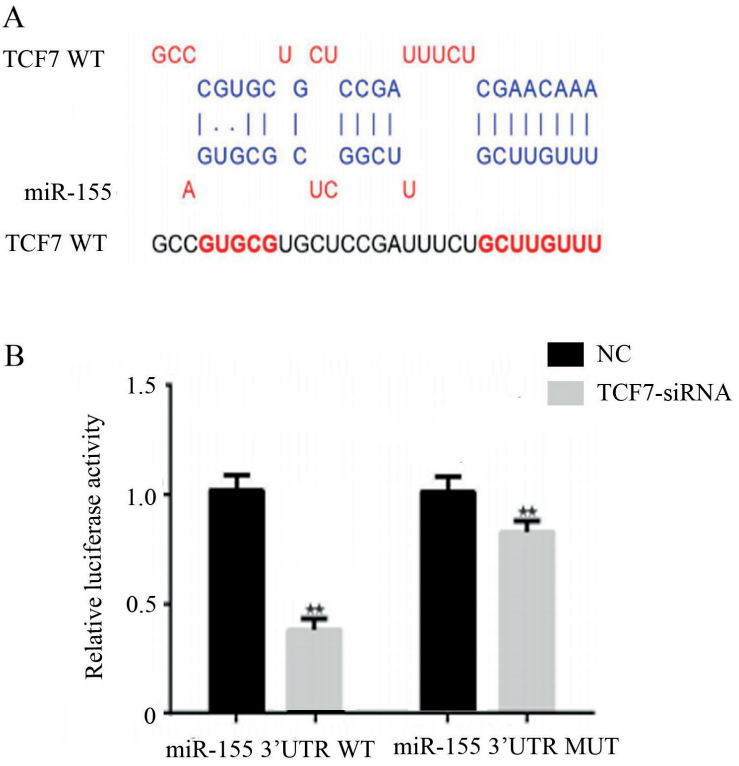

Figure 3. Dual-Luciferase assay to detect the relationship between TCF7 and miR-155 in cervical cancer cells. **A,** Bioinformatics to detect the relationship between TCF7 and miR-155. **B,** Dual-Luciferase assay to detect TCF7 can regulate the activity of miR-155.

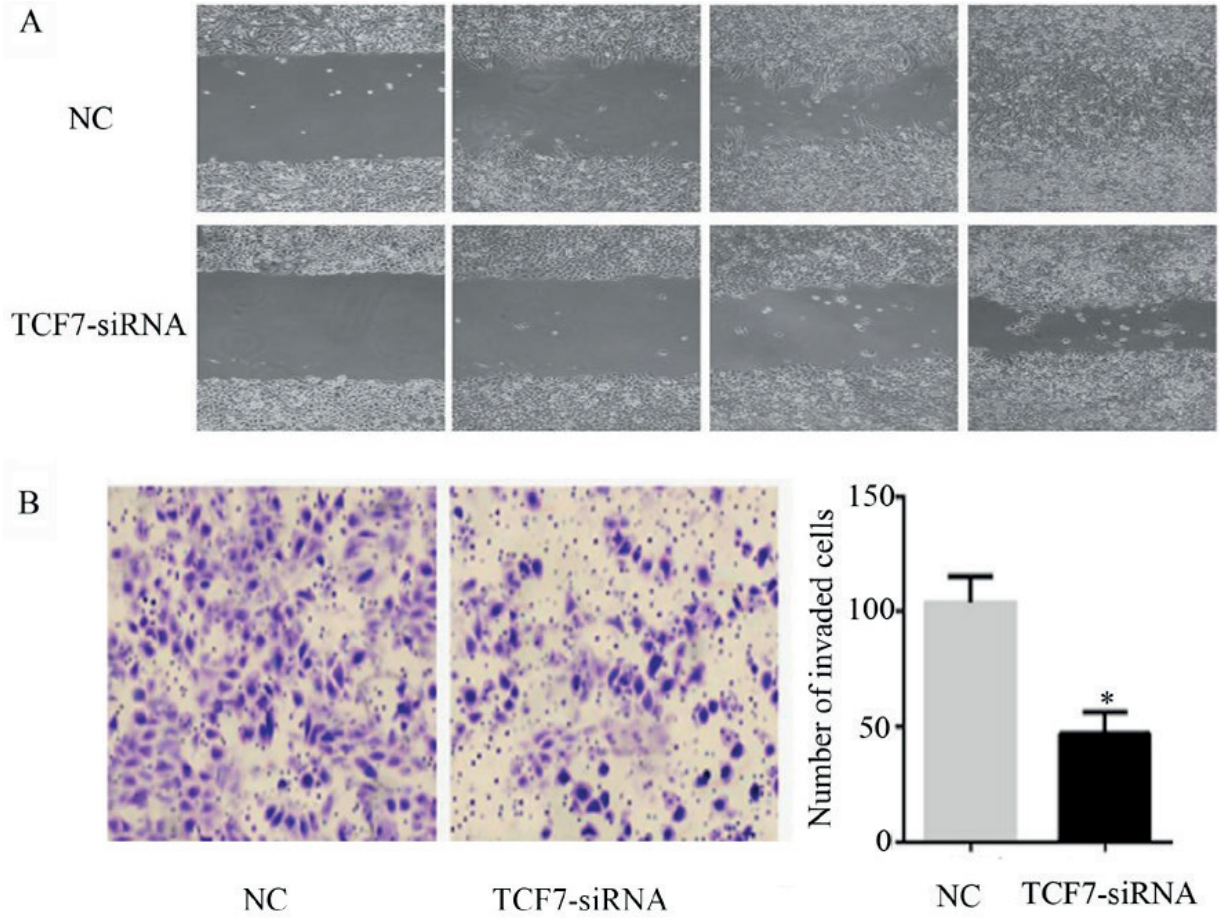

Figure 4. Effect of TCF7 on migration and invasion behavior of cervical cancer cells. **A,** Effect of TCF7 on migration behavior of cervical cancer cells. **B,** Effect of TCF7 on the invasion behavior of cervical cancer cells. (Magnification 40 ×).

cells. Therefore, the overexpression of miR-155 can reverse the inhibitory effect of TCF7 on the migration and invasion of cervical cancer cells, indirectly indicating that there is a mutual regulation between miR-155 and TCF7.

Effect of LncRNA TCF7 on Tumor Formation in Nude Mice

Tumor formation in nude mice showed that the average tumor volume and mean tumor weight of the TCF7-siRNA group were significantly lower than those of the control group [tumor volume (0.28±0.04) *vs.* (0.97±0.11), $p<0.05$; tumor weight (0.21±0.03) *vs.* (0.93±0.15), $p<0.05$], the results showed that down-regulation of LncRNA TCF7 can attenuate the proliferation of cervical cancer cells in nude mice (Figure 6).

Discussion

MiR-155 is one of the miRNAs that are significantly down-regulated due to the HPV infection[18]. In addition to cervical cancer, miR-155 can also act as a tumor suppressor and down-regulated in other malignant tumors, such as colorectal cancer[19], gastric cancer[20], hepatocellular carcinoma[21], and breast cancer[22]. However, its mechanism of down-regulation in cervical cancer cells is not well understood. Kong et al[23] have reported that methylation-mediated transcriptional repression is a possible mechanism for the down-regulation of miR-155 in cervical cancer cells. Therefore, we decided to further study the details of miR-155 transcriptional repression in cervical cancer cells.

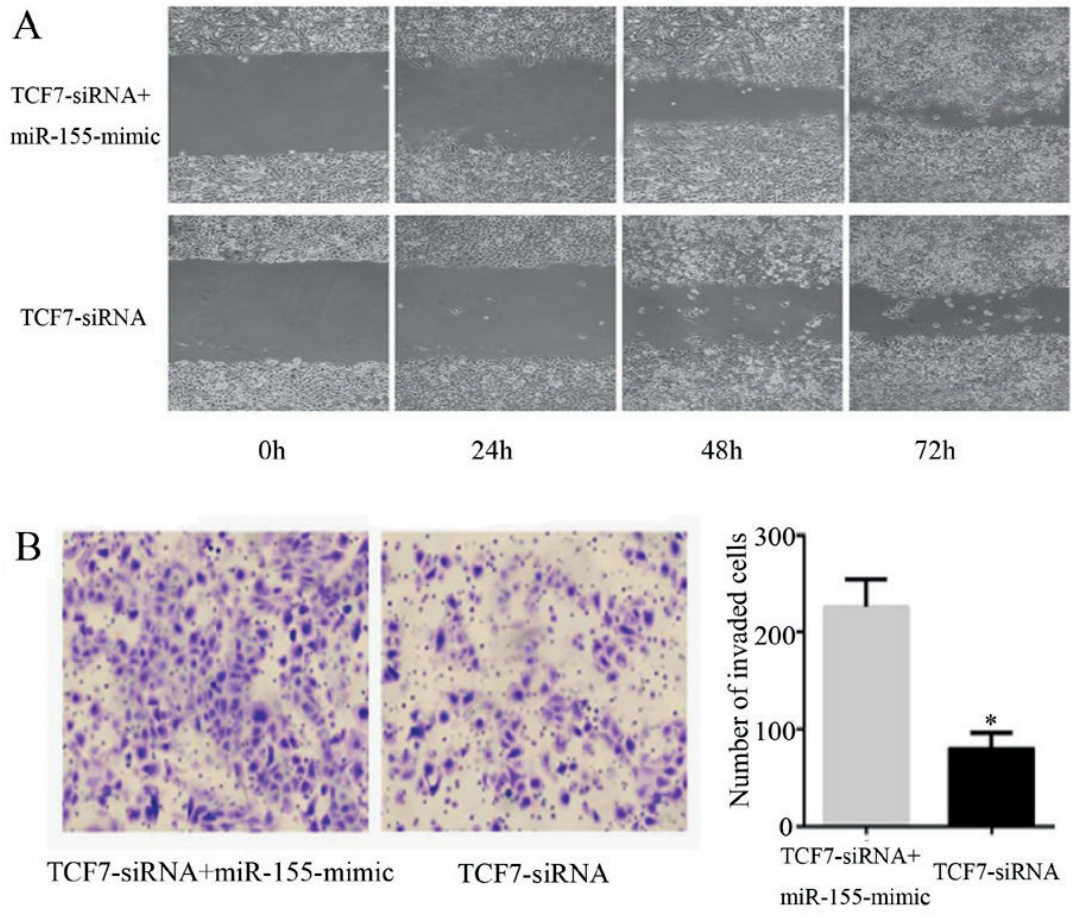

Figure 5. Effect of miR-155 on migration and invasion behavior of cervical cancer cells. **A,** Effect of miR-155 on migration behavior of cervical cancer cells. **B,** Effect of miR-155 on invasion behavior of cervical cancer cells. (Magnification 40 ×).

Expression of HR-HPV infection and viral oncoproteins can lead to a series of dysregulation of biophysical processes. HPV-16 E6 and E7 proteins regulate the methylation of tumor suppressor genes such as MT1G, NMES1, RRAD, SFRP1, SPARC, and TFPI2 in SiHa and CaSki cells, and inhibit the degradation of DNMTs by E6 and E7 oncogenes[24]. Another study[25] observed that the knockdown of E6 in HPV-16-positive human cervical cancer SiHa and CaSki cells directly led to inhibition and promotion of DNMT1 protein. In this report, it was further confirmed that HPV-16 E6 can positively regulate DNMT1 expression in SiHa and CaSki cells. The knockdown of DNMT1 restored the level of miR-155 in the cells. Therefore, we conclude that miR-155 is down-regulated due to DNMT1-mediated activation of promoters in HPV-16-positive cervical cancer cells.

In the past few years, the concept of competing endogenous RNA (ceRNA) had been proposed to explain the new regulatory mechanisms of RNA, which explains that RNA can crosstalk by competing to share miRNAs, thereby regulating the bioavailability of miRNAs. TCF7 has previously been identified as a carcinogenic lncRNA factor, at least by acting as a miRNA regulator in several types of cancers. TCF7 can modulate the ZEB2 expression by stimulating endogenous RNA in spongy miR-200s in clear cell renal cell carcinoma[26]. It also allows miR-124 to bind and increase the expression of GRB2, a downstream gene of miR-124, and promote the growth and invasion of HR-HPV-positive cervical cancer cells[27]. According to previous studies[28,29], both miR-155 and TCF7 are involved in the regulation of epithelial-mesenchymal transition in a variety of cancers. However, any association between miR-155 and TCF7 is unclear. Bioinformatics analysis revealed a corresponding putative binding site with miR-155 and the Dual-Luciferase assay confirmed the

regulatory relation therein. QRT-PCR analysis showed that the inhibition of TCF7 expression significantly reduced miR-155 expression, while miR-155 overexpression simultaneously reversed inhibition of TCF7 levels. Thus, these results indicate that TCF7 and miR-155 form a functional axis of regulatory biology in cervical cancer. Zargar et al[30] reported that miR-155 directly targeted HPV-16 E6 mRNA and reduced its protein levels, while HPV-16 E6 enhanced TCF7 expression in cervical cancer cells. Combined with the results of this experimental report, it can be inferred that there is negative feedback regulation between TCF7 and E6 through DNMT1, and DNMT1 forms part of the TCF7, miR155, and HPV-16 E6 networks in cervical cancer cells.

Conclusions

This work found the expression and regulation of lncRNA T5CF-7 and miR-155 in cervical cancer cells. Functionally, the knockdown of lncRNA TCF-7 can inhibit the invasiveness of cervical cancer cells *in vitro* and *in vivo*, and the overexpression of miR-155 can reverse its effects to some extent. This investigation provides a new regulation strategy for the molecular mechanism and clinical treatment of cervical cancer.

Conflicts of interest

The authors declare no conflicts of interest.

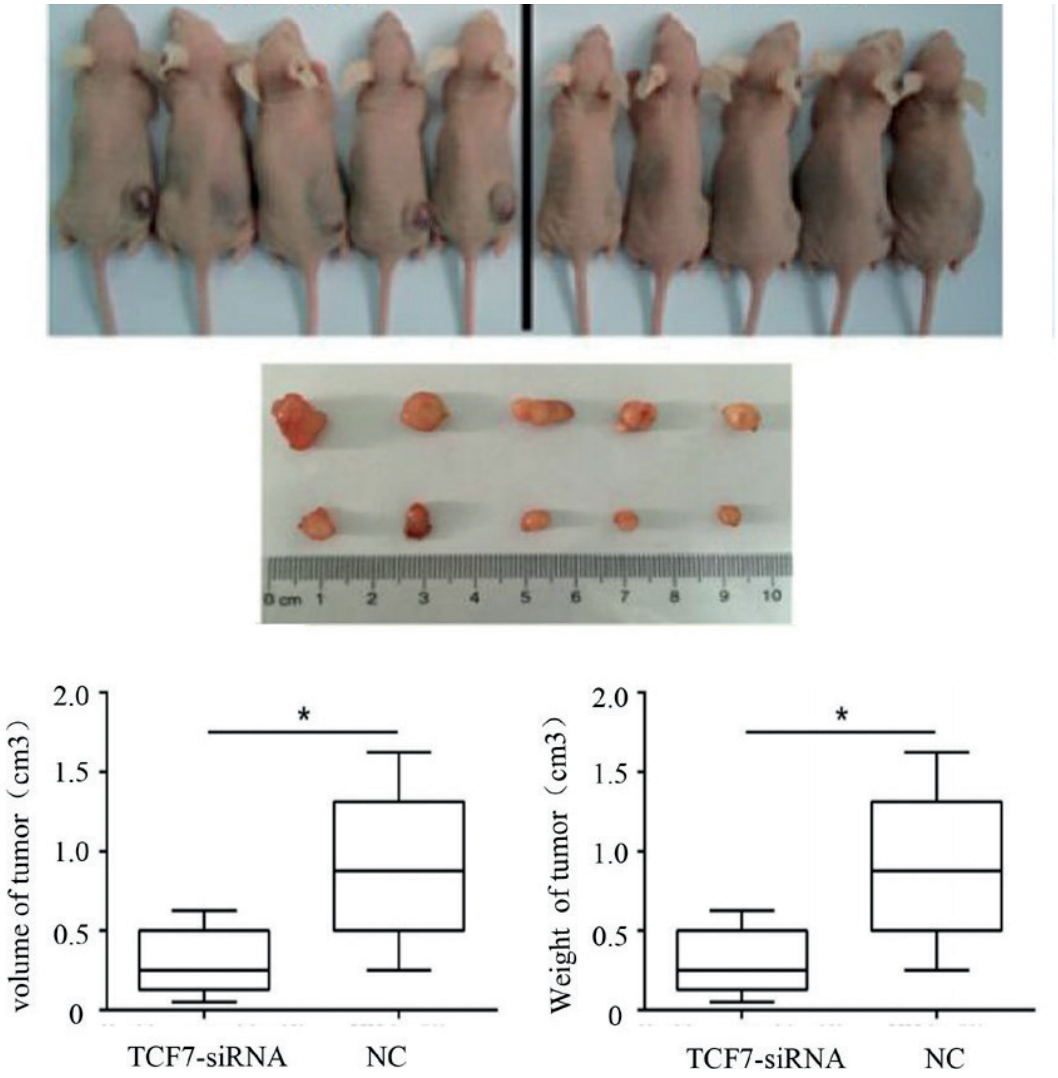

Figure 6. Effect of LncRNA TCF7 on tumor formation in nude mice.

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