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MicroRNA-143 (miR-143) Suppresses Cell Proliferation and Invasion by Downregulating AKT/STAT3/NF-κB Pathway in Tongue Squamous Cell Carcinoma (TSCC)

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ABSTRACT

Background: MicroRNA-143 (miR-143) has been reported that its biological function is to serve as a target to inhibit tumor progression in human oral squamous cell carcinoma (OSCC). However, its role and molecular mechanisms in tongue squamous cell carcinoma (TSCC) were still unknown.

Methods: Several *in vitro* experiments were performed using CAL-27 cells, including cell proliferation experiment, wound healing experiment, flow cytometry, transwell and colony formation experiments. In addition, we also performed *in vivo* experiments to study the potential application of miR-143 in tumorigenesis of TSCC.

Result: The result exhibited that miR-143 up-regulation could significantly suppress proliferation, migration and invasion ability of CAL-27 cells and make these cells stop at G0/G1 phase. We reported that upregulation of miR-143 would suppress its tumorigenic ability. Moreover, we also found that the overexpression of miR-143 would reduce the phosphorylation level of STAT3, p65 and AKT. In addition, miR-143 overexpression decreases CDK2 expression while as expression level of E-cadherin was increased.

Key words: AKT/STAT3/NF-κB, miR-143, Tongue squamous cell carcinoma, Tumor progression.

INTRODUCTION

The proportion of tongue squamous cell carcinoma (TSCC) in oral malignant tumors is reported to be between 25% and 40% globally (Kimple *et al.*, 2014). Some risk factors, including drinking alcohol, smoking and human papillomavirus infection, were thought to be related to the development of TSCC (El-Husseiny *et al.*, 2000; Ramqvist *et al.*, 2015). Although most researchers have made many improvements and efforts in the mainstay treatment for TSCC, including surgical resection, adjuvant chemotherapy or radiotherapy, the five-year overall survival rate remains below 55%. Therefore, elucidating the molecular mechanisms underlying TSCC progression helps optimize treatment and improves TSCC survival status.

As a group of short non-coding RNA, microRNA (miRNAs/miRs) have currently been identified to play a suppressive or promotive role in TSCC progression (Yu and Li, 2016). Sun and colleagues (Sun *et al.*, 2016) reported that overexpression of miR-137 could act as tumor suppressor to inhibit the tumor progression in TSCC cells. Shi *et al* (Shi *et al.*, 2018) indicated that miR-488 overexpression could significantly suppress the invasion and epithelial-mesenchymal transition of TSCC cells. Inversely, miR-21-5p may have the function to enhance tumor proliferation and improve their invasion ability; also suppress TSCC cell apoptosis through PI3K/Akt/FOXO1 pathway (Liu *et al.*, 2019). In particular, miR-143 is significantly down-regulated in OSCC and was correlated with advanced tumor size (Mayakannan *et al.*, 2015). Subsequently, it was further demonstrated that up-regulation of miR-143 decreases OSCC cell migration, glucose metabolism and proliferation and increase apoptosis and make cells stop at G0/G1 phase (Sun and Zhang, 2017). However, the biological function and regulating mechanisms of miR-143 in TSCC remains unclear.

Considering TSCC accounts for approximately 80% of subtypes of OSCC (Ramqvist *et al.*, 2015), we speculated miR-143 possibly act as a suppressive molecule in TSCC. Thus, we performed this research to study its function on TSCC cell migration, invasion, proliferation and adhesion ability. We also validated its function on tumor growth *in vivo*. Moreover, possible role of miR-143 in regulating AKT/STAT3/NF-κB pathway in TSCC cells was studied.

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MATERIALS AND METHODS

Cell culture and transfection

CAL-27 TSCC cell was obtained from American type culture collection and cultured in an incubator set at 5% CO_2 and

37°C using DMEM. MiR-143 mimics and its control (miR-NC) were provided by Shanghai GenePharma Co. Ltd. (Shanghai, China). For cell transfection, 2 × 10^5 CAL-27 cells were added into six-well plates per well and transfected with 50 nM of miR-143 mimics or normal control using Lipofectamine 2000 (Invitrogen). CAL-27 cells without any transfection were used as blank group.

All the experimental work was carried out in The General Hospital of Western Theater Command (Chengdu, China) between July 2020 to January 2022).

Cell proliferation

3000 transfected CAL-27 cells were added into each well of 96-well plates and were cultured overnight. After that, we add 10 ul CCK-8 solution into each cell and incubated them for 2 h at 37°C at day 1, 3, 5 and 7 respectively. Then, the optical density (OD) value was measured at 450 nm wavelength.

Colony formation experiment

Transfected CAL-27 cells (500 cells per well) were added into a six-well plates. Two weeks later, we fixed those colonies using 4% paraformaldehyde and then stained 30 minutes in crystal violet (0.1%). Subsequently, those single colonies were counted within three random fields.

Cell cycle analysis

Transfected cells at 48 h were digested with trypsin and re-suspended with PBS. After fixed with 75% cold ethanol and stay overnight at 4°C and PBS washing then cells were incubated 30 minutes within 100 µL propidium iodide solutions in darkness. The proportion of cells at different cell cycle stages was detected using a Flow Cytometer (BD Biosciences).

Cell adhesion assay

Transfected CAL-27 cells (1×10^4) were added into Matrigel pre-coated 96-well plates (2 µg per well). After incubation at 37°C for 24 hours and those floating cells washed away, then we use 4% formaldehyde to fix those remaining cells and stained it with crystal violet (0.1%). Finally, cell adhesion rates were determined based on the absorption at 595 nm using an enzyme-labeling reader (Bio-Tek Instruments, VT, USA).

Wound healing experiment

After the transfected cells grew to 90% confluence, a vertical line was drawn using a pipette tip. After 24, 48 and 72 h incubation, respectively, series of photographs were taken using the microscope across the drawn line and then the open wound area was calculated using Image-Pro software (National Institutes of Health, version 6.0).

Transwell assay

Transfected cells were adjusted to a density of 5 × 10^4 cells/mL using DMEM without serum and then added into the upper chamber (Merck Millipore, Billerica, MA) pre-coated with 100 µL/well Matrigel and there were 600 µl DMEM containing 10% FBS in the lower chamber. 24 hours later, we fixed and stained those migrated cells in the lower chamber and then photographed under a microscope. Those cells remain in the upper chamber were rinsed.

Quantitative real-time PCR

RNA of CAL-27 cells was distracted from transfected using TRIzol method and then reversed to cDNA with PrimeScript RT Reagent Kit (Takara). After reverse transcription, quantitative PCR was carried out using SYBRGreen (Takara) with appropriate primers designed by Primer 5.0 according to the conditions, including an initial step of 10 minute in 95°C and then 40 cycles of amplification, which includes 10 s in 95°C, 20 s in 60°C and 30 s in 72°C. The mRNA level of target gene was determined by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2000). The internal control used was GAPDH and the experiment was repeated three times.

Tumor xenograft model

Female 4-5-week-BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All the mice were provided free access to tap water and diet *ad libitum*. Then, 5 × 10^6 transfected CAL-27 cells or normal control cells or blank cells were injected into the back flanks of mice subcutaneously. On the 28th day, the volume of tumors were calculated following this formula: 0.5×length×width^2(mm^3). Meanwhile, the mice were sacrificed and the tumors were preserved for further experiments after sacrifice those mice in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry

Those preserved tumors underwent paraffin embedding and were sliced into 4 µm-sections. Then these sections were heated at 60°C overnight and dewaxed in xylene. After dehydrated with gradient ethanol, the sections were blocked in 10% normal goat serum and incubated with primary antibodies of CDK2 and E-cadherin and stay at 4°C for one night and then stained with DAB and hematoxylin. Different degrees of staining slices were obtained using microscope (Olympus).

Western blot

Cell or tumor tissue protein was distracted using RIPA buffer (Sigma-Aldrich, USA) and quantification was finished using BCA protein assay kit. Protein electrophoresis separation was performed using SDS-PAGE gel (12%) and then transferred to PVDF membranes and protein blocking using 5% skim milk. After that, membranes were incubated in the primary antibodies against CDK2, E-cadherin, p-AKT, p-STAT3, p-p65 and beta-actin (all from Abcam, Cambridge, UK) and 4°C overnight, followed by secondary antibodies 37°C for 2 hours. The protein signals were detected through chemiluminescence kit and furthermore analysed by using ImageJ software.

RESULTS AND DISCUSSION

Up-regulation of miR-143 inhibits cell proliferation, cell adhesion, migration and invasion

First of all, we evaluated the function of miR-143 on cell proliferation, cell cycle distribution and cell adhesion. The results from the above experiment indicated that the cell proliferation and cell adhesion was suppressed after miR-143 up-regulation, as shown in (Fig 1). In addition to proliferation, metastasis is another important characteristic of OSCC cells. Next, we evaluated the function of miR-143 on CAL-27 cell migration and invasion ability. The results from the wound healing experiment indicated that the wound area was decreased after miR-143 mimics-transfection at 24, 48 and 72 h, respectively (Fig 2A). In other words, miR-143 overexpression shortened the migration distance of CAL-27 cells. Consistently, the number of invasive cells was significantly decreased after miR-143 overexpression (Fig 2B). Therefore, miR-143 up-regulation could inhibit the migration and invasion ability of TSCC cells.

Consistent with our data, miR-143 mimics transfection could significantly inhibit proliferation of lung cancer cell (Wei *et al.*, 2015) and invasion and migration ability of prostate carcinoma cell (Wu *et al.*, 2013). In addition, miR-143 exerted its suppressive effects on nasal squamous cell carcinoma (Qian *et al.*, 2019), colon cancer (Liu and Liu, 2019) and pancreatic ductal adenocarcinoma (Xie *et al.*, 2019).

About the association between miR-143 and OSCC, several studies have been reported as follows: (Bufalino *et al.*, 2015) reported that down-regulation of miR-143 was associated with activin A overexpression involved in increased proliferation and invasiveness in OSCC cells. It has been confirmed that transfection of miR-143 mimics could significantly lead to G1 stage arrest and induce cell

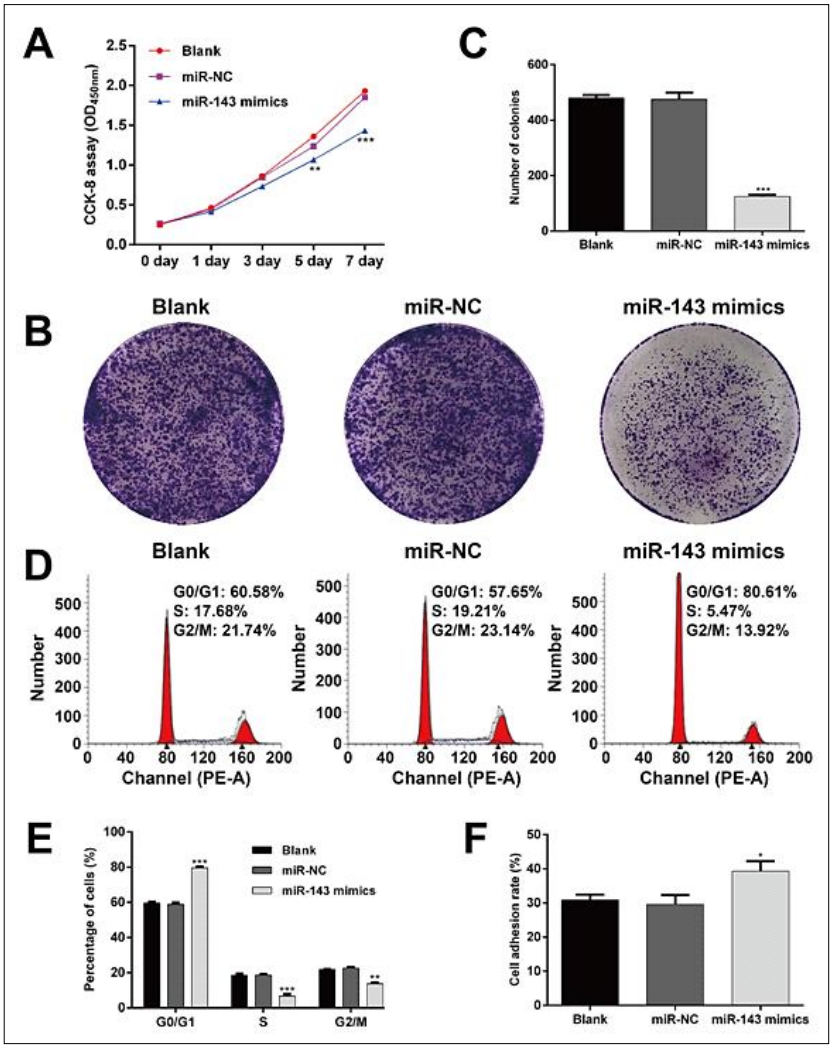

Fig 1: Results of miR-143 up-regulation on TSCC cell proliferation, cell cycle distribution and cell adhesion. (A) The proliferation of transfected CAL-27 cells and normal control group was detected using CCK-8 method. (B-C) The colony formation ability was decreased after the up-regulation of miR-143. (D-E) The proportion of cells at different stages of cell cycle was determined using flow cytometry. (F) Cell adhesion rate of two groups was analyzed. Data were shown as mean ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

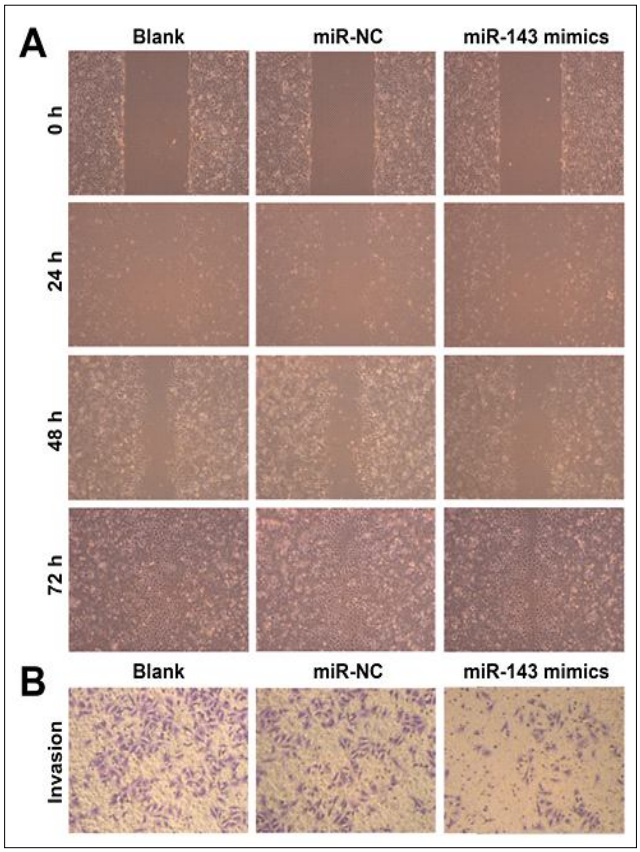

Fig 2: Influence of miR-143 overexpression on TSCC cell invasion and migration. (A) Scratch wound healing experiment results of two groups. (B) Migration and invasion experiment results of transfected CAL-27 cells and normal control.

apoptosis in the oral cancer cells (OECM-1 and Tca8113) (Sun and Zhang, 2017). Consistently, Xu *et al* (Xu *et al.*, 2015) reported that miR-143 exhibited a significant function as the tumor-suppressive gene to the CAL-27 cell migration and invasion, which was not the same as our study that further presented the influence of miR-143 on tumor progression and cell cycle *in vivo.*

Up-regulation of miR-143 suppressed the AKT/STAT3/ NF-κB pathway and tumor growth in mice

We studied the function of miR-143 on AKT/STAT3/NF-κB pathway molecules, both the expression and activation level in CAL-27 cells (Fig 3A-B). Compared with the other two groups, the phosphorylation of the AKT, STAT3 and p65 in CAL-27 cells were lower at both mRNA and protein levels (Fig 3B-C).

We established xenograft models using transfected CAL-27 cells and normal control cells to elucidate the tumor-suppressive role of miR-143. Image of the tumors in the nude mice showed that the tumor size of mice treated with miR-143 mimics was smaller than that tumor of other groups (Fig 4A). The result also showed the tumor growth was suppressed after miR-143 transfection, as reflected by decreased tumor volume in the miR-143 mimics group in comparison with other groups (Fig 4B). These results suggested that miR-143 could suppress the growth of TSCC cells.

Subsequently, tumors were excised for immunohisto chemical staining for the CDK2 and E-cadherin expression. Cells positive of CDK2 was decreased, while E-cadherin-positive cell number in the miR-143 mimics group was higher than the other two groups (Fig 5A). Meanwhile, expression

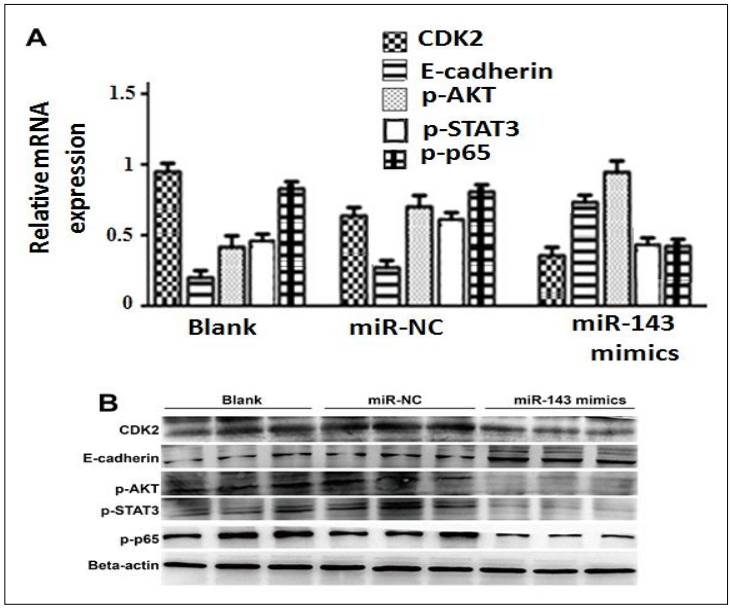

Fig 3: Influences of miR-143 overexpression on AKT/STAT3/NF-κB pathway in TSCC cells. (A) The mRNA levels of CDK2, E-cadherin, AKT, STAT3 and NF-κB in two groups. (B) The protein levels of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 in two groups.

of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 was detected in tumor tissues. According to the result of western blotting, miR-143 overexpression down-regulated the protein level of CDK2, p-AKT, p-STAT3 and p-p65, while up-regulated E-cadherin protein expression (Fig 5B-C). These data indicated that miR-143 inhibited TSCC tumor cell growth partly by down-regulating the AKT/STAT3/NF-κB pathway.

At the molecular level, it was reported that miR-143 up-regulation suppressed the protein level of CDK2, p-AKT, p-STAT3 and p-p65, while up-regulated E-cadherin protein expression *in vitro* and *in vivo*. Cyclin-dependent kinase (CDK) 2 is a crucial regulatory molecule essential for G1-S transition (Guadagno and Newport, 1996). Here, miR-143 induced cell cycle G0/G1 phase arrest might be associated

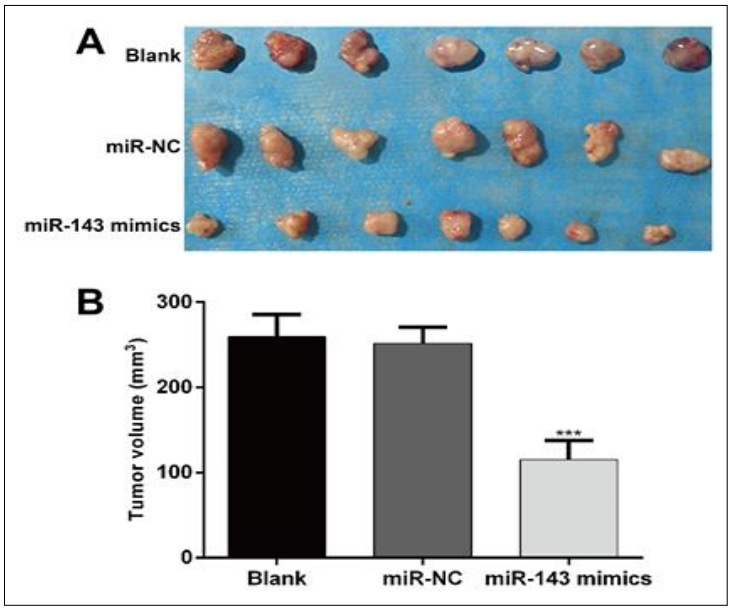

Fig 4: Tumor xenotransplantation model was used to confirm tumor suppression function of miR-143. (A) Representative images of subcutaneous tumors. (B) The volume of subcutaneous tumors for three groups.

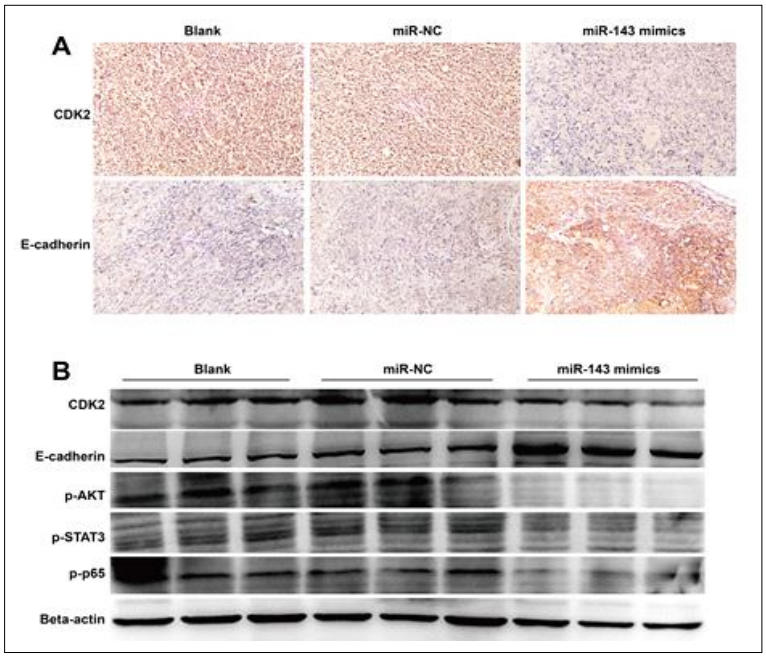

Fig 5: Influences of miR-143 overexpression on AKT/STAT3/NF-κB pathway *in vivo*. (A) Immunohistochemistry staining of CDK2 and E-cadherin in xenograft tumors derived from miR-143- miR-NC- or blank-transfected CAL-27 cells. (B) Western blotting was applied to determine the expression levels of CDK2, E-cadherin, p-AKT, p-STAT3 and p-p65 in xenograft tumors derived from miR-143- miR-NC- or blank-transfected CAL-27 cells.

with the down-regulation of CDK2. During the process of tumor progression, tumor cells obtain more efficient invasive and migratory abilities were called epithelial-to-mesenchymal transition (EMT). E-cadherin is one of the most common markers of EMT, which mainly mediates the loss of cell-cell adhesion, was down-regulated and would promote cell migration and invasion (Puisieux *et al*., 2014). Here, the increased E-cadherin induced by miR-143 overexpression might be correlated with suppressed cell migration and invasion ability in miR-143 mimics-transfected CAL-27 cells. As the core of the PI3K/Akt pathway, Akt is essential for cell survival and growth, which down-regulation could suppress tumor cell proliferation (Wang *et al*., 2019). Signal transducer and activator of transcription 3 (STAT3) have been reported to promote tumor cells proliferation, survival and tumor invasion (Chai *et al*., 2016). Activation of NF-κB is associated with increased cell cycle progression, cell migration and invasion (Shishodia *et al*., 2005). The result of our experiments indicated that miR-143 overexpression down-regulates the level of p-AKT, p-STAT3 and p-p65. According to the literature research, miR-22 overexpression could inhibit PI3K/Akt/NF-κB pathway to promote apoptosis in TSCC cells (Gu *et al*., 2018). Additionally, Cryptotanshinone exerted anti-proliferative effects on TSCC cells by down-regulating the level of p-STAT3 and CDK2 and up-regulation of E-cadherin (Wang *et al*., 2017). We thus propose that miR-143 could suppress CAL-27 cells proliferation and migration by down-regulate the phosphorylation level of AKT, STAT3 and p65.

CONCLUSION

In conclusion, the study presented evidences *in vitro* and *in vivo* that up-regulation of miR-143 would significantly downregulates the invasion and proliferation ability of CAL-27 cells and could also induce cell cycle G0/G1 phase arrest. We further demonstrated the evidence of AKT/STAT3/NF-κB pathway for the first time in participating in miR-143 exerting suppressive effects in TSCC cells. These discoveries would lay a solid foundation for future novel gene therapy strategies of TSCC.

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None

Ethics approval

This study has gotten the approval of the Ethics Committee at General Hospital of Western Theater Command and all experiments were conducted according to the Declaration of Helsinki.

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Conflicts of interest

The authors declare that no conflict of interest exists.

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