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Original Article

miR-222 knockdown suppresses epithelial-to-mesenchymal transitionin human oral squamous cell carcinoma

Ying Ouyang*, Fangfang Jiang*, Binghui Zeng, Changbo Wei, Dongsheng Yu

*Guangdong Provincial Key Laboratory of Stomatology, Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou 510055, Guangdong, P.R. China. *Equal contributors.*

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Abstract: Tumor metastasis is the main cause of death in patients with oral squamous cell carcinoma (OSCC). Epithelial-to-mesenchymal transition (EMT) is potentially associated with metastasis and histological grading in OSCC. Therefore, the discovery of new strategies to inhibit EMT is potentially valuable for the development of therapies for OSCC. In our previous study, we found that miR-222, which is up-regulated in OSCC, regulates the biological behavior of OSCC cells by targeting the p53-upregulated modulator of apoptosis (PUMA); however, the effect of miR-222 on TGF-β1-induced EMT in OSCC cells is unclear. In this study, OSCC cell lines CAL-27 and Tca-8113 were incubated with 5 ng/ml of TGF-β1 to inhibit the expression of E-cadherin, promote the expression of N-cadherin, vimentin, and α-SMA and stimulate achange in cell shape convert from a "cuboidal" epithelial structure into an elongated mesenchymal shape. We found that the expression of miR-222 was up-regulated during TGF-β1-induced EMT in OSCC cells. In addition, miR-222 knockdown reversed TGF-β1-induced EMT by targeting PUMA. Our findings indicate that miR-222 plays an important role in OSCC, potentially serving as a novel therapeutic target for the treatment of OSCC.

Keywords: miR-222, PUMA, EMT, TGF-β1, oral squamous cell carcinoma

Introduction

Oral cancer, a type of head and neck cancer of the oral cavity, is one of the most common cancers worldwide [1] and is recognized as a global public health threat. Smoking, alcohol use, and human papillomavirus (HPV) infection are the major risk factors for oral cancer, with smoking and alcohol consumption exerting synergistic effects [2]. In china, betel quid chewing contributes significantly to the risk of oral cancer [3]. Oral squamous cell carcinoma (OSCC), which represents more than 90% of oral cancers, is the most frequent of all cancers of the oral cavity [4]. Although local OSCC maybe effectively controlled by surgical excision and radiotherapy, once metastasis has occurred, no effective treatment is available and the mortality rate is significantly elevated [5]. Tumor metastasis is the main cause of death in patients with oral cancer. The invasion of tumor cells is a complex, multistage process.

Epithelial-mesenchymal transition (EMT) is a crucial event required for the dissemination of cells from epithelial tumors: in malignant epithelial cancers, epithelial cells lose their polarity and acquire a mesenchymal phenotype; this is followed by detachment from the basement membrane, which facilitates migration [6, 7]. Multiple pleiotropic cytokines and several signaling pathways are involved in this process. In addition, the expression of epithelial markers E-cadherin is down-regulated and the expression of mesenchymal markers V-cadherin, vimentin, and α-SMAis up-regulated during EMT. EMT is potentially associated with metastasis and histological grading in OSCC [8]. The inhibition of EMT by over-expressing miR-204 and P120 ctn has been shown tosuppress the migration and invasion of cancer cells in OSCC [9, 10]. Therefore, the development of strategies targeting EMT is necessary for successfully treating OSCC.

Table 1. Primer sequences for qRT-PCR

Primer	Primer sequence (5'-3')
E-cadherin Forward	CCCACCACGTACAAGGGTC
E-cadherin Reverse	CTGGGGTATTGGGGGCATC
N-cadherin Forward	CTAATGGTCGGCGTATCTACT
N-cadherin Reverse	CGTAAGATGGAGGAACATCA
Vimentin Forward	CGCCAGATGCGTGAAATGG
Vimentin Reverse	ACCAGAGGGAGTGAATCCAGA
α-SMA Forward	GTGAAGCAGCTCCAGCTATG
α-SMA Reverse	CGTCCCAGTTGGTGATGATG
18s rRNA Forward	CCTGGATACCGCAGCTAGGA
18s rRNA Reverse	GCGGCGCAATACGAATGCCCC
hsa-miR-222 Reverse Transcription	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGACCCAGTA
hsa-miR-222 Forward	ACACTCCAGCTGGGAGCTA-CATCTGGCTACTG
hsa-miR-222 Reverse	CTCAACTGGTGTCGTGGA
U6-Reverse Transcription	AACGCTTCACGAATTTGCGT
U6 Forward	CTCGCTTCGGCAGCACA
U6 Reverse	AACGCTTCACGAATTTGCGT

MicroRNAs (miRNAs) aresmall non-coding RNA molecules, containing approximately 22 nucleotides, that suppress gene expression bybinding directly to the mRNA 3'-UTR [11, 12]. Previous studies have shown that miRNAs regulate the expression of various oncogenes and tumor suppressors that play important roles in metastasis and tumor progression in OSCC [13, 14]. Recent research has shown that miR-221/222 promotes EMT in breast cancer andnon-small cell lung cancer [15, 16]. In our previous study, we found that miR-222, which was up-regulated in OSCC, regulates the migration and invasion of OSCC cells by targeting p53-upregulated modulator of apoptosis (PUMA) [17]; however, the effect of miR-222 on EMT in OSCC cells is unclear. In this study, we aimed to clarify the effect of miR-222 on TGF-β1-induced EMT in OSCC cells, and to elucidate the underlying molecular mechanism.

Materials and methods

Cell culture and stimulation

The OSCC cell lines CAL-27 and Tca-8113 were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, Carlsbad, CA, USA). All cells were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. For experiments, CAL-27 and Tca-8113 cells were cultured in serum-free media containing 0.1% bovine serum albumin for 24 h before treated with 5 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ, USA).

RNA isolation and quantitative real-time (qRT-PCR)

Tca-8113 and CAL-27 cells were harvested and lysed using Trizol reagent (Invitrogen, CA, USA). In order toanalyze miR-222 expression, reverse transcription PCR (RT-PCR) was performed using specific stem-loop reverse transcription primers; miR-222 first strand synthesis was performed using a First Strand Synthesis Kit (Takara, Dalian, China), and qPCR was performed using a Mir-X™ miRNAqRT-PCR SYBR® Kit (Takara, Dalian, China). U6 was used as an internal control. In order to quantify mRNA levels of E-cadherin, N-cadherin, vimentin, α-SMA, PUMA, and phosphatase and tensin homolog (PTEN), RT-PCR was performed using the PrimeScript RT Reagent Kit with cDNA Eraser (Takara, Dalian, China) and qPCR was performed using SYBR Premix Ex Taq (Ta-kara, Dalian, China), with 18s rRNA as an internal control. The primer sequences used for qRT-PCR are shown in **Table 1**. Gene expression was analyzed using the Applied Biosystems 7500 system (Applied Biosystems, Warrington, UK), measured in triplicate, quantified using the $2^{-\Delta\Delta CT}$ method, and normalized to that of a control.

Western blot

The protein levels of E-cadherin, N-cadherin, vimentin, α-SMA, PUMA, and PTEN were determined by western blot. Protein extracted from Tca-8113 and CAL-27 cells was centrifuged at 14,000 *g* for 20 min at 4°C. The protein concentration was determined using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were resolved by SDS-PAGE on a 10% gel and transferred to a PVDF membrane. Membranes were blocked with 5% nonfat milk in TBS for 3 hours and incubated with a 1:1,000 dilution of anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-α-SMA, anti-PUMA, and anti-PTEN (all obtained from Cell Signaling

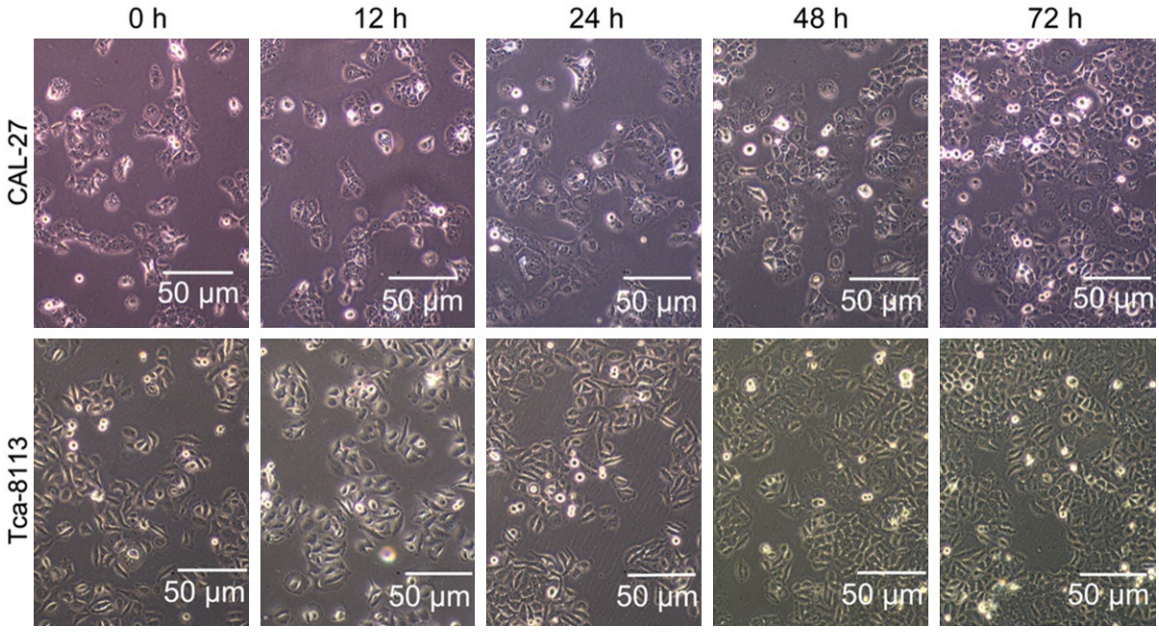

Figure 1. Alternations of cell shape during TGF-β1-induced EMT in CAL-27 and Tca-8133 cells after stimulated with TGF-β1. Cell shape was monitored by phase contrast microscope during EMT, which was induced by treatment of 5 ng/ml TGF-β1 in OSCC cell lines CAL-27 and Tca-8113 (200×).

Technology, Beverly, MA, USA) at 4°C overnight, followed by incubation for 40 min with a 1:20,000 dilution of secondary antibody (BOSTER, Wuhan, Hubei, China). Proteins were visualized using ECL (Thermo Scientific Pierce ECL Plus, Thermo Scientific, Rockford, IL, USA). GAPDH was used as a loading control for comparison between samples.

Plasmid construction and transfection

A control (miR-NC) and miR-222 inhibitor were purchased from Jima Biotech (Suzhou, China). Cells were plated at 50% confluence and transfected with 300 nM miR-NC, or miR-222 inhibitorusing Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the recommended protocol. After transfection,cells were stimulated with 5 ng/ml TGF-β1 and used for subsequent analysis.

Migration and invasion assay

Cell migration and invasion were assessed using a transwell migration assay. For migration assay, CAL-27 and Tca-8113 cells were harvested and 5×10^4 cells, in 200 μL of 0.1% serum medium, were placed into the upper chamber of an insert (pore size, 8 μm) (BDBiosciences, San Diego, CA, USA). The lower chamber was filled with 10% FBS medium (600 μL). For invasion assays, 5×10^4 cells were seeded into anupper chamber pre-coated with Matrigel (BD Biosciences, San Diego, CA, USA), and the lower chamber was filled with 10% FBS medium (600 μL). Cells were incubated for 24 h and then removed from the upper chamber with a cotton swab. Next, the cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution in 20% ethanol. Migrationand invasion of cells were observed using a LEICA microscope at 200× magnification, in five independent fields for each well, and the average counts were calculated.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software (IBM Inc., USA). Continuous variables are presented as means ± standard deviation (SD). A t-test was used to compare the differences between groups; $P<0.05$ were considered to represent statistically significant differences.

Results

TGF-β1-induces EMT in OSCC cells

OSCC cells CAL-27 and Tca-8113 were incubated with 5 ng/ml of TGF-β1 to examine wh-

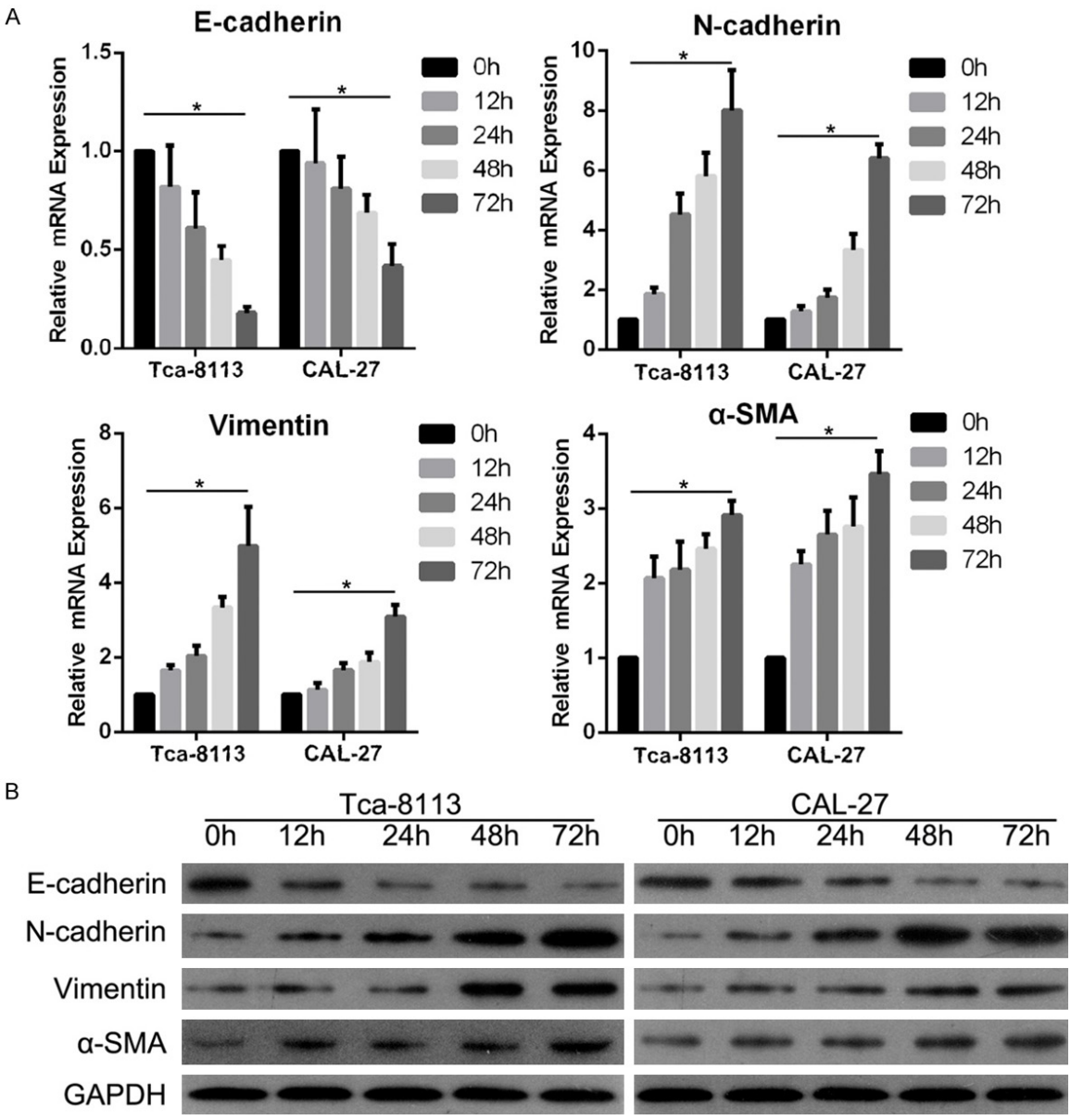

Figure 2. Expressions of the EMT-associated proteins in Tca-8113 and CAL-27 OSCC cell exposed to TGF-β1 treatment. A: After stimulated with TGF-β1 for 24 h, the expression of E-cadherin, N-cadherin, vimentin, and α-SMA were analyzed by qRT-PCR. The results are presented as means ± SD. **P*<0.05. B: After stimulated with TGF-β1 for 24 h, the expressions of E-cadherin, N-cadherin, vimentin, α-SMA and GAPDH were detected by western blot.

ether TGF-β1 stimulates EMT. Stimulation by TGF-β1 resulted in a change of cell shape from a "cuboidal" epithelial structure into an elongated mesenchymal shape, as time progressed (**Figure 1**). EMT was a developmental process in which epithelial cells acquire migratory characteristics. qRT-PCR and Western blot results (**Figure 2**) showed that TGF-β1 stimulation down-regulated the expression of epithelial markers E-cadherin and up-regulated the expression of mesenchymal markers N-cadherin, vimentin, and α-SMA significantly (*P*<0.05). TGF-β1-induced EMT ina time-dependent manner in OSCC cell lines.

TGF-β1 promotes miR-222 expression and inhibits PUMA expression

Emerging evidence revealed that miR-222 played critical regulatory roles in cell metastasis and development; therefore, we studied the expression levels of miR-222 during TGF-β1-induced EMT in OSCC cells using qRT-PCR. The results showed that TGF-β1 stimulation result-

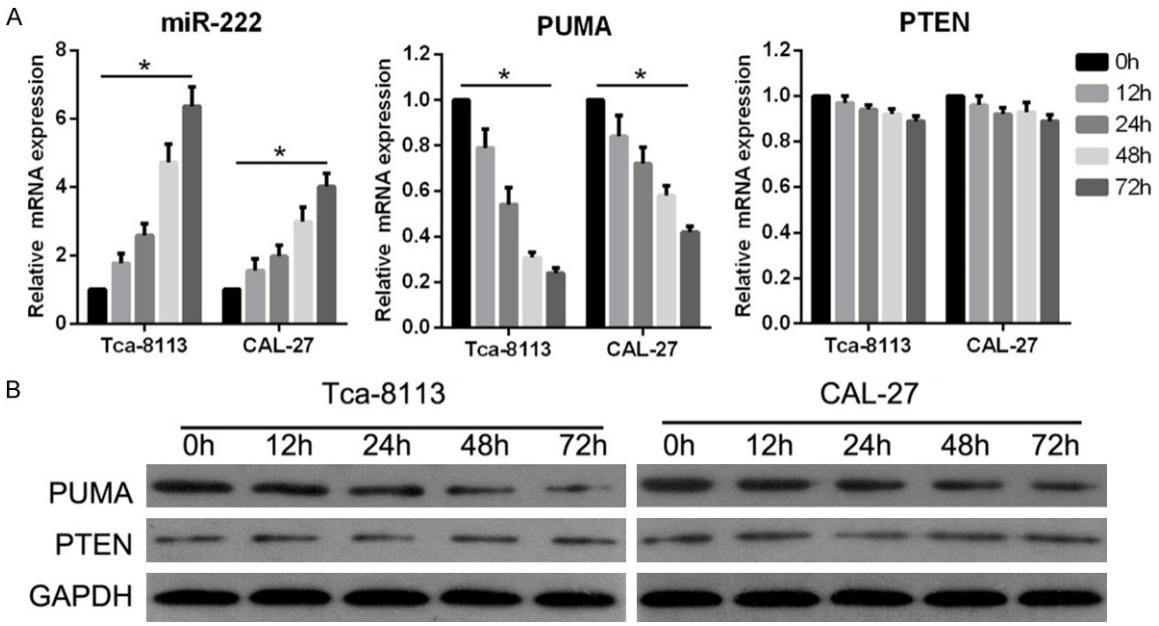

Figure 3. The expressions of miR-222, PUMA and PTEN in Tca-8113 and CAL-27 exposed to TGF-β1 treatment. A: After stimulated with TGF-β1 for 24 h, the expression of miR-222, PUMA, and PTEN was studied by qRT-PCR; the results are presented as means ± SD; **P*<0.05. B: After stimulated with TGF-β1 for 48 h, the expression of PUMA and PTEN was detected by western blot.

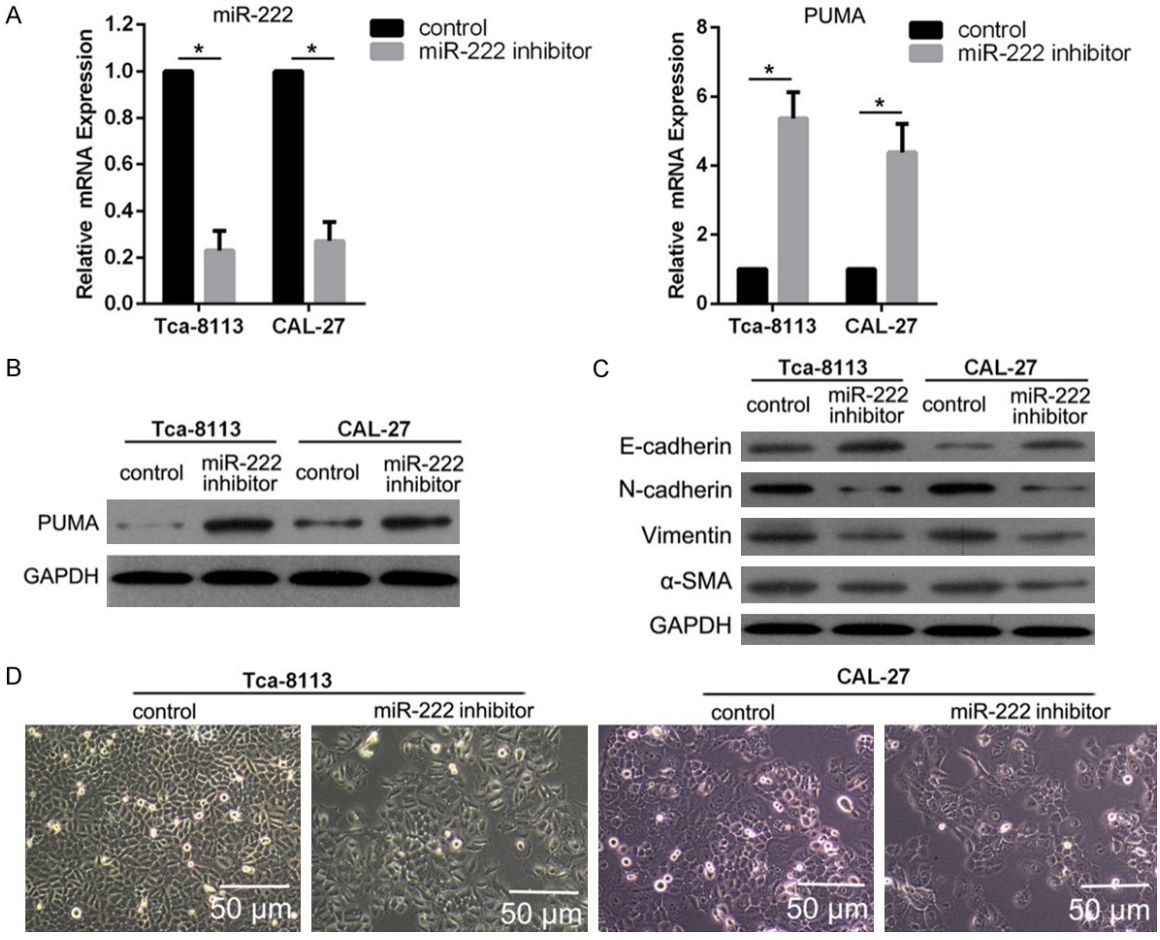

Figure 4. miR-222 inhibitor inhibited PUMA expression and EMT in Tca-8113 and CAL-27 exposed to TGF-β1 treatment. A: After transfection, Tca-8113 and CAL-27 cells were stimulated with 5 ng/ml TGF-β1 for 24 h and the expression of miR-222 and PUMA was detected using qRT-PCR. Results were presented as means ± SD. **P*<0.05 *vs* control group. B: After transfection, Tca-8113 and CAL-27 cells were stimulated with 5 ng/ml TGF-β1 for 48 h and the expression of PUMA was studied using western blot. C: After transfection, Tca-8113 and CAL-27 cells were stimulated with 5 ng/ml TGF-β1 for 48 h and the expression of E-cadherin, N-cadherin, vimentin, and α-SMA was detected by western blot. D: After transfection, Tca-8113 and CAL-27 cells were stimulated with 5 ng/ml TGF-β1 for 24 h and cell shape was analyzed in Tca8113 and CAL-27 cells (200×).

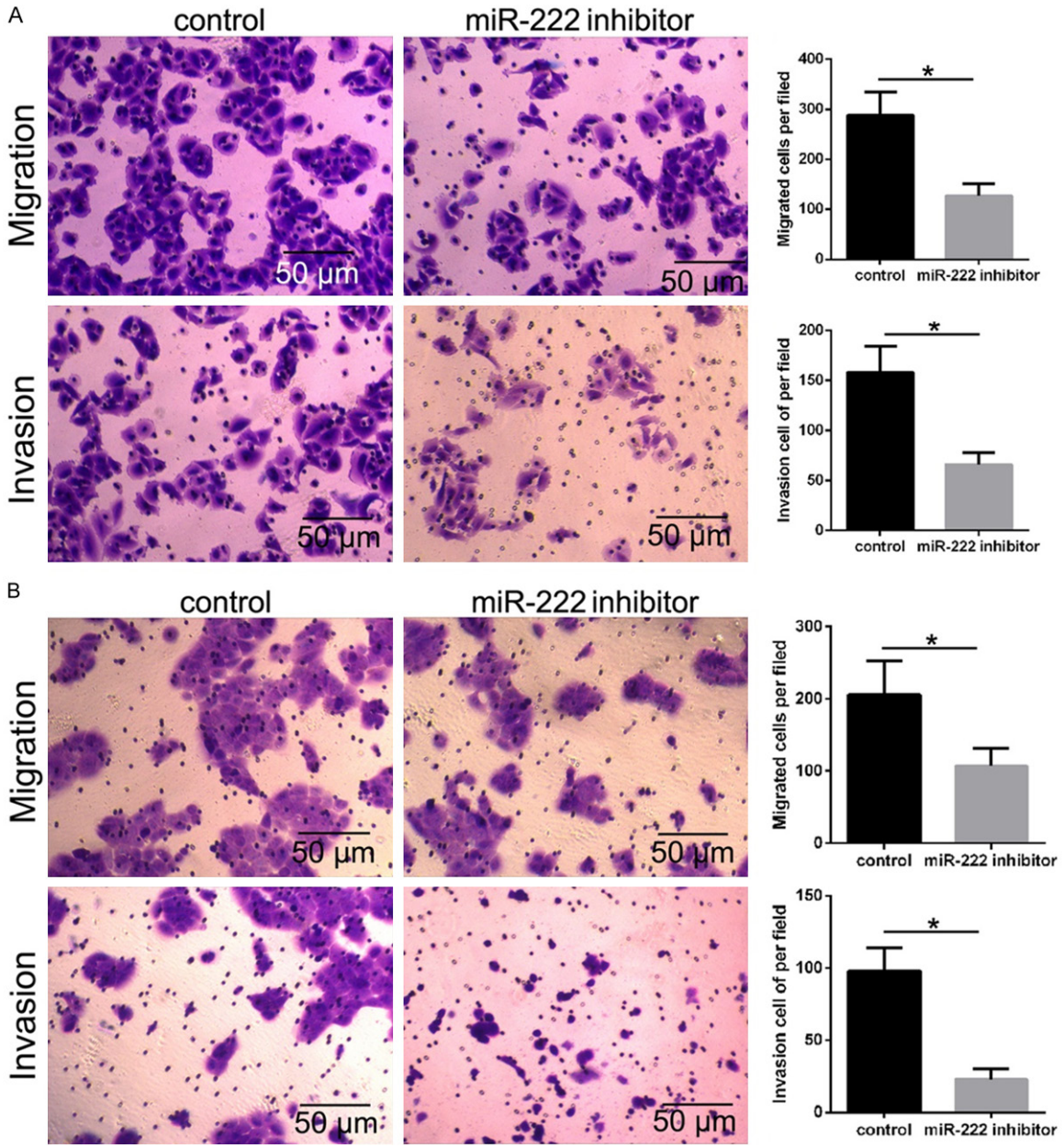

Figure 5. miR-222 inhibitor inhibited migration and invasion of Tca-8113 and CAL-27 cellsexposed to TGF-β1 treatment. A: After transfection, Tca-8113 cells were stimulated with 5 ng/ml TGF-β1 for 48 h and were examined migration and invasion. miR-222 inhibitor inhibited the migration and invasion of Tca-8113. B: After transfection, CAL-27 cells were stimulated with 5 ng/ml TGF-β1 for 48 h and were examined migration and invasion. miR-222 inhibitor inhibited the migration and invasion of CAL-27. Results were presented as means ± SD. **P*<0.05 *vs* control group.

ed in asignificantincrease in the expression of miR-222 in CAL-27 and Tca-8113 cells (P<0.05), and the effect was found to be time-dependent (**Figure 3A**). *PUMA* and *PTEN* represent potential targets of miR-222 in OSCC cells; accordingly, we determined the expression levels of the respective genes and encoded proteinsin CAL-27 and Tca-8113 cell lines by qRT-PCR and Western blot (**Figure 3A** and **3B**). The results showed that the expression level of PUMA was significantly decreased in CAL-27 and Tca-8113 cells (P<0.05); however, no differences in the expression level of PTEN were observed. The results showed that, during TGF-β1-induced EMT, miR-222 expression was significantly up-regulated and PUMA expression was significantly down-regulated.

miR-222 inhibitor reverses TGF-β1-induced EMT by targeting PUMA

In order to elucidate the role of miR-222 during TGF-β1-induced EMT, CAL-27 and Tca-8113 cells were transfected with miR-222 inhibitor and stimulated with TGF-β1. The transfected miR-222 inhibitor was found to effectively decrease the expression of miR-222 (P<0.05, **Figure 4A**) and increase PUMA expression (P<0.05, **Figure 4A** and **4B**). The results showed that miR-222 maybe negatively correlated with PUMA expression in OSCC cell lines and indicated that the transfection of miR-222 inhibitor effectively increased the expression of E-cadherin and decreased the expression of N-cadherin, vimentin, and α-SMA (P<0.05, **Figure 4C**). Stimulation with TGF-β1 resulted in a change of cell shape from a “cuboidal” epithelial structure into an elongated mesenchymal shape. However, with the transfection of miR-222 inhibitor, the change of cell shape was effectively suppressed (**Figure 4D**).

miR-222 inhibitor inhibits the migration and invasion of OSCC cells

After transfection, CAL-27 and Tca-8113 cells were stimulated with 5 ng/ml TGF-β1 for 48 h and were examined migration and invasion of cells. The transwell assay showed that the migration and invasion of cells were significantly inhibited in miR-222 inhibitor transfected cells compared with the control group (P<0.05) (**Figure 5**).

Discussion

In this study, we found that OSCC cells incubated with 5 ng/ml of TGF-β1 could down-regulate the expression of epithelial markers E-cadherin and up-regulate the expression of mesenchymal markers N-cadherin, vimentin, and α-SMA significantly. We also found that stimulation by TGF-β1 resulted in a change of cell shape from a “cuboidal” epithelial structure into an elongated mesenchymal shape, and induced EMT in OSCC cells. The TGF-β signaling pathway regulated various target genes to govern multiple biological processes during tumor progression. TGF-β activates both Smad-dependent and Smad-independent pathways to function as a potent extracellular inducer of EMT [18, 19]. In OSCC, TGF-β1-induces EMT and promotes metastasis and bone invasion [20, 21], which were similar to those of our study.

Recent research has shown that miR-222 promotes EMT in cancer cells [15]. The expression of miR-222 is elevated in gastric cancer and prostate cancer, as well as in other types of cancer [22, 23]. High expression of miR-222 is correlated with shorter metastasis-free survival, lower 5-year survival rates, and lower overall survival [22]. The knockdown of miR-222 inhibits cell growth and invasion, and increases radiosensitivity [24]. In previous study, we found that the expression of miR-222 is elevated in OSCC tissues [17]. In this study, we detected that the expression of miR-222 is up-regulate during TGF-β1-induced EMT in OSCC cells. The present findings, which were similar to those of our previous study, indicate that miR-222 may play an important role in TGF-β1-induced EMT in OSCC cells.

miR-222 plays an important role in the occurrence and development cancers by directly binding to its target mRNA 3’-UTR to regulate gene expression. Numerous target genes of miR-222, such as *PTEN* (in gastric cancer and prostate cancer) [23, 24], p27 (in breast cancer) [25], and *ARID1A* (in cervical cancer) [26] have been identified. miR-222 overexpression enhances proliferation and invasion, decreases apoptosis, and reduces sensitiz ation to cisplatin by targeting PUMA in OSCC [17, 27]. We additionally studied the expression of *PTEN* and *PUMA*, both of which were target genes of miR-222 in OSCC. The tumor suppressor PTEN regulates cell proliferation, migration, and angiogenesis via phosphatidylinositol phosphatase, which in turn regulates the activation of AKT via PI3K [28]. PUMA was newly discovered as a target for activation by p53 to promote cell apoptosis through binding to and neutralizing

pro-survival members of the Bcl-2 family [29]. In this study, we found that TGF-β1-induced EMT in OSCC cells was accompanied by the down-regulation ofthe expression of PUMA; however, that of PTEN was not found to change. When miR-222 expression was inhibited during TGF-β1-induced EMT, the expression of PUMA was up-regulated. These results show miR-222 might target PUMA during TGF-β1-induced EMT and the present findings were similar to those ofour previous study In addition, we found that the inhibition of miR-222 resulted in significant down-regulation of the expression of N-cadherin, vimentin, and α-SMA and up-regulation of the expression of E-cadherin, preventing the change of cell shape from a "cuboidal" epithelial structure into an elongated mesenchymal shape, reversing TGF-β1-induced EMT, and inhibit the migration and invasion of OSCC cells.

In conclusion, miR-222 knockdown suppresses TGF-β1-induced EMT andour findings strongly indicated that miR-222 plays an important role in OSCC, and may serve as a novel therapeutic target for the treatment of this cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dongsheng Yu, Guangdong Provincial Key Laboratory of Stomatology, Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou 510055, Guangdong, P. R. China. E-mail: yudsh@mail.sysu.edu.cn

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