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TGF-β1 promotes cells invasion and migration by inducing epithelial mesenchymal transformation in oral squamous cell carcinoma

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Abstract. – OBJECTIVE: To investigate the role of TGF-β1 on epithelial mesenchymal transition (EMT) and invasion in oral squamous cell carcinoma cell line Tca8113.

MATERIALS AND METHODS: Cultured Tca8113 cells were treated with different concentrations of TGF-β1 for 24 h. The morphological changes were observed under phase-contrast microscopy. The mRNA and protein expression levels of EMT relative marker E-cadherin and Vimentin were detected by RT-PCR and Western blot. The effect of TGF-β1 on migration and invasion ability of Tca8113 cells were detected using transwell method.

RESULTS: The results demonstrated that TGF-β1 could induce morphological changes in Tca8113 cells from epithelial to mesenchymal. The mRNA and protein level of epithelial marker E-cadherin was downregulated following treatment with TGF-β1, whereas the mRNA and protein expression level of mesenchymal marker protein Vimentin was upregulated. Furthermore, TGF-β1 significantly enhances the migration and invasiveness of Tca8113 cells, which were effectively reversed by TGF-β1 inhibitor, LY2109761

CONCLUSIONS: TGF-β1 enhances Tca8113 cells migration and invasion by inducing epithelial mesenchymal transition.

Key Words:
Oral squamous cellcarcinoma, Epithelial mesenchymal transformation, Transforming growth factor, Invasion.

Introduction

As one of the most common malignant tumors in head and neck, oral carcinoma accounts for 3% of overall incidence of general tumors with an average annual increase of 100,000 patients worldwide[1]. Oral squamous cell carcinoma (OSCC), a kind of epithelial invasive tumor, is the most frequent oral malignant tumor comprising of 90% of the oral carcinoma cases[2]. Currently, combination therapy of surgery, chemotherapy and radiotherapy is used to treat OSCC. However, the survival rate of the patients with OSCC is only 50% due to the high-degree of malignancy of OSCC and the susceptibility to develop invasion or distant metastasis. Therefore, to investigate the mechanisms of occurrence, development, invasion and metastasis of OSCC is of great significance for improving the prognosis of patients.

The invasion and metastasis of tumors are regulated by multiple factors and involve multiple sequential procedures. In recent years, epithelial mesenchymal transition (EMT) has become a hot topic in tumor research. An increasing number of scholars in China and other countries are focusing on the application of EMT in the research of tumor invasion and metastasis. EMT refers to a process in which the epithelial characteristics of the epithelial cells are replaced by interstitial characteristics under some special physiological or pathophysiological conditions[3,4]. In the process of EMT, the migration and motor ability of the epithelial cells are enhanced, and the phenotype of epithelial cells is transformed into that of the interstitial cells. This process is accompanied by the loss of polarity and connection with surrounding cells, the decrease in exposure to the cell matrix and alterations in the cytoskeleton. Not only does the ETM participate in the processes of early development of embryo and organogenesis, but it is also involved in the processes of various epithelial malignant tumors by promoting invasion and metastasis of the cells[5].

As a kind of polypeptide cytokines with multiple biological effects are excreted by various cells, the transforming growth factor can exert important regulatory effects in the processes of cell proliferation and apoptosis; it is also involved in some important biological processes, e.g. angiogenesis, repair of fibrosis injury and the occurrence of tumor[6,7]. The TGF-β signaling pathway has become one of the widely-accepted signal pathways mediating and regulating the EMT process[8,9].

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In this study, we aimed to investigate EMT induced by TGF-β1 in human Tca8113 cells of OSCC.

Materials and Methods

Materials

Tca8113 cells of human OSCC (Institute of Biochemistry and Cell Biology, SIBS, CAS); TGF-β1 (R&D, USA); TGF-β1 inhibitor, LY2109761 (Selleck, Shanghai, China); TRIzol RNA extraction reagent (TaKaRa, Otsu, Shiga, Japan); primers synthesized by Shanghai Tsingke Biology Co., Ltd.; E-Cadherin rabbit mAb and goat anti-mouse IgG or goat anti-rabbit IgG (HRP-conjugate, Cell Signaling, Danvers, MA, USA); Vimentin mouse anti-human mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA); protein marker (Thermo Scientific, Waltham, MA, USA); ECL chemiluminescent kit (Millipore, Billerica, MA, USA); calf serum (Gibco, Grand Island, NY, USA); Roswell Park Memorial Institute 1640 (RPMI-1640) media (HyClone, GE Healthcare Life Sciences, Logan, UT, USA); cell lysate (radioimmunoprecipitation assay – RIPA) and BCA protein quantitation kit (Beyotime, Shanghai, China); 24-well transwell with pore size of 8 μm (Corning Costar, New York, NY, USA); Matrigel for invasion experiment (BD, China).

Cell Culture

Tca8113 cells were cultured in the RPMI-1640 media supplemented with calf serum (10%), penicillin (100 U/mL), and gentamicin (100 μg/mL) in the incubator (37°C, 5% CO_2, and saturated humidity). The cells were digested using trypsin for passaging. Cells were cultured until the degree of cell fusion reached 70% to 80%. Thereafter, they were starved with serum-free media overnight then treated with TGF-β1 for 24h. The cells were divided into groups according to the experiment requirement.

Morphologic Observation of Cells

Tca8113 cells were cultured overnight on 6-well plates in serum-free media; Cells which adhered to the surface were treated with TGF-β1 for 48h and then observed for cell morphology using phase contrast microscope.

RNA Extraction and Real-time PCR Detection

TGF-β1 treated cells were washed for 3 times on ice with precooled PBS (phosphate buffered saline); 1 mL of Trizol RNA extraction buffer was added into each well; the total RNA of cells was extracted following manufacturer's instructions. The ratio of A_{260}/A_{280} was detected using ultraviolet spectrophotometr and RNA concentration was calculate in triplicate. The reverse transcription from the extracted RNA to cDNA was performed using RT kit and cyclic amplification by PCR was conducted for the acquired cDNA. The sequences used for PCR were synthesized by the Shanghai Branch of Invitrogen (Carlsbad, CA, USA), and the sequences of primers were shown as follow: GAPDH upstream primer 5'-ATGGGGAAGGTGAAGGTCG-3', and downstream primer 5'-GGGTCATTGATGGCAACAATATC-3'; β-catenin upstream primer 5'-GCTGCTGTTTTGTTCCGAATGT-3', and downstream primer 5'-GCCATTGGCTCTGTTCTGAAGA-3'; Vimentin upstream primer 5'-CCAAACTTTTCCTCCCTGAACC-3', and downstream primer 5'-GTGATGCTGAGAAGTTTCGTTGA-3'; E-cadherin upstream primer 5'-GTACTTGTAATGACACATCTC-3', and downstream primer 5'-TGCCAGTITCTGCATCTGC-3'. The PCR conditions included a pre-denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 1 min and final extension at 72°C for 10 min. Electrophoresis in agarose gel (1.5%) was performed for the PCR products and the gray scale was scanned by gel imaging system.

Western Blot Detection

After 48h of treatment by TGF-β1 in various concentrations (0 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL), cells adherent to culture flasks were washed by pre-heated PBS. cells were transferred into tubes (Eppendorf, Hamburg, Germany) and centrifuged at 12000 rpm for 5 min. Cell lysates were collected and supernatants were preserved at -20°C. Samples were run on electrophoresis at 80V to accumulate the proteins, which were later isolated at 100V then transferred to the membrane. The membrane was blocked using 5% skim milk for 1h and then incubated overnight at 4°C with the addition of E-Cadherin rabbit anti-human mAb (1:1000), Fibronectin mouse anti-human mAb, Vimentin rabbit anti-human PcAb (1:100) and p-AKT mouse anti-human mAb (1:500). The membrane was then washed by TBS-T (Tris Buffered Saline-Tween) on the shaker for 3 times (5 min/wash). Goat anti-mouse IgG or goat anti-rabbit IgG (HRP-conjugate) was added to the membrane and incubated for 1h at room temperature. The membrane was washed and incubated with ECL chemiluminescent reagent for 1 min at room

temperature, and the membrane were exposed, fixed and developed by X-ray image.

Cell Migration and Invasion Experiment

1. **Cell migration experiment:** Tca8113 cells at the logarithm phase were starved in the RPMI (Roswell Park Memorial Institute)-1640 medium for 24h, then digested with 0.25 trypsin-EDTA solution and the RPMI-1640 serum-free media was prepared for the single-cell suspension with the cell density being adjusted to 3×10^5/mL and more than 95% of cell viability assayed by trypan blue staining. 200μL of single-cell serum-free suspension was added into the upper chamber of the transwell; meanwhile the drugs were added into each group with 3 complex pores. 600 μL RPMI-1640 media containing 10% of calf serum was added into the lower chamber of the transwell, and transwells were incubated in the incubator for 24h. Thereafter, chambers were taken out and washed by PBS twice to remove the media. The residual cells in the upper chamber were wiped by wet cotton swab followed by 20 min of fixation by methanol. Cells were dried at the room temperature, and then stained for 20 min using crystal violet. The stained plate was placed under the inverted microscope for counting the number of cells passed through the membrane, i.e. the number of cells spreading in the central and surrounding visions (400×) and the average was calculated.
2. **Cell invasion experiment:** The Matrigel was melted at 4°C in advance and diluted with serum-free media (1:3); 40 μL of the diluted Matrigel was spread over the membrane on the polycarbonate micro-pores and then was placed in the incubator for 4h to coagulate; the concentration of inoculated cells was regulated to 2×10^5/mL, and the rest procedures were exactly the same as with the cell migration experiment.

Statistical Analysis

All the data were presented as $\chi \pm$ SD (mean ± standard deviation). One-way variance analysis was performed for statistical analysis using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered as statistically significant.

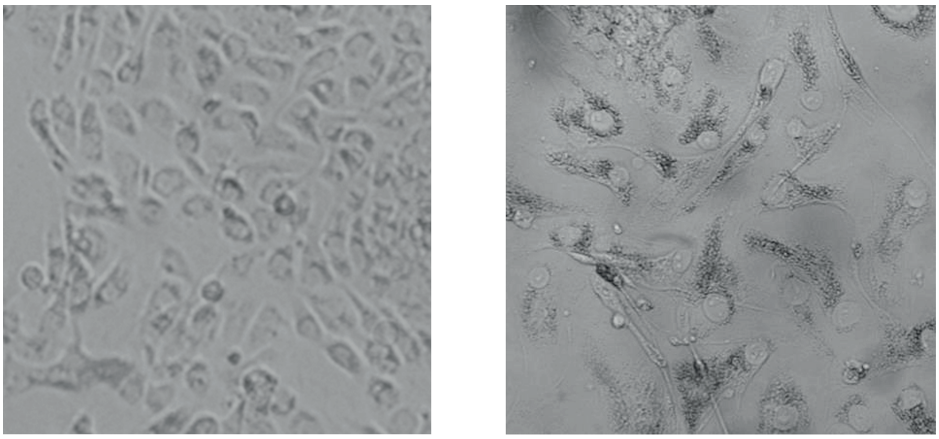
Results

The Effect of TGF-β1 on Promoting the Transformation from Epithelioid Tca8113 Cells to Mesenchymal-like

After Tca8113 cells were stimulated by 10 ng/mL of TGF-β1 for 24h, the photographs taken by phase contrast microscope showed that the Tca8113 cells in the control group presented as typical epithelioid structure, while in the TGF-β1 stimulation group, we found most of the cells were significantly stretched like the elongated spindle with the obvious characteristics of the interstitial cells (Figure 1).

The effect of TGF-β1 on Upregulating Vimentin mRNA Expression and Downregulating E-cadherin mRNA Expression

After the cells were treated with various concentrations of TGF-β1 (0 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL) for 24h, RT-PCR results showed stimula-



0 ng/mL TGF-β1 10 ng/mL TGF-β1

Figure 1. Morphologic changes of Tca8113 cells induced by 10 ng/mL of TGF-β1 for 24h.

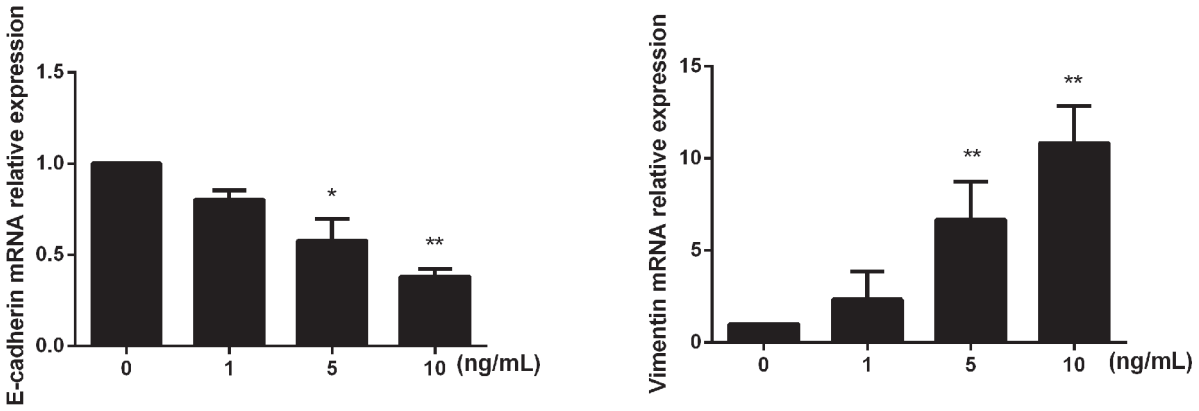


Figure 2. The mRNA expression level of E-cadherin and Vimentin of Tca8113 cells induced by different concentrations (0 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL) of TGF-β1 for 24 h.

tion by TGF-β1 decreased the mRNA expression of E-cadherin in a dose dependent manner, while the mRNA expression of Vimentin, was increased (Figure 2). The difference between groups was statistically significant ($p<0.05$)

The Effect of TGF-β1 on Upregulating Vimentin Protein Expression and DownRegulate E-cadherin Protein Expression

After the cells were treated by TGF-β1 in various concentrations (0 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL) for 24h, Western blot results showed that stimulation by TGF-β1, decreases the expression of E-cadherin protein, while an increase in the protein expression of Vimentin was observed, in a dose dependent manner (Figure 3). The difference between different groups was statistically significant ($p<0.05$)

TGF-β1 Inhibitor Reverses the Transformation from Epithelial Cells to the Interstitial Cells

After the cells were treated with 10 ng/mL of TGF-β1 for 24h, the mRNA and protein expressions of E-cadherin in the cells with epithelial phenotype were significantly decreased, and significant increases were seen in those cells with interstitial phenotype ($p<0.05$). However, this EMT process was reversed when 10 ng/mL of the LY2109761, the TGF-β1 inhibitor was added ($p<0.05$) (Figure 4).

TGF-β1 Promotes the Migration and Invasion of Tca8113 Cells

In the experiment of Transwell migration, we found that after being treated by TGF-β1 for 24h, the Tca8113 cells in the TGF-β1 stimulatedgroup showed significantly enhanced ability to migrate compared to the control group ($p<0.05$) (Figure 5A). In the experiment of Transwell migration, we found that after being treated by TGF-β1 for 24h, the Tca8113 cells in the TGF-β1 stimulation group showed significantly enhanced ability in invasion compared to the control group ($p<0.05$) (Figure 5B).

Discussion

OSCC is the most common malignant tumor in head and neck, and the occurrence and development of OSCC are regulated by multiple factors and consist of multiple steps with various biological characteristics such as high-degree malignancy, rapid local growth and susceptibility to invasion, metastasis and recurrence in advanced stage. Thus, making an early diagnosis, blocking the occurrence and the development of tumors as well as giving the effective treatment have become the key to prophylaxis and treatment for OSCC.

The metastasis of malignant tumor is a process involving the mutual regulation among multiple genes and factors, including the following processes: tumor cells detaching from the original focal; migration of these cells by the blood circulation to some place far away from the original focal and formation of the metastatic tumor was by colonization, in which the formation is completed by sequential procedures of local infiltration, hematogenous metastasis and cell proliferation[10]. More and more studies have confirmed the wide participation of EMT in the biological metastasis and invasion processes of multiple tumors, in-

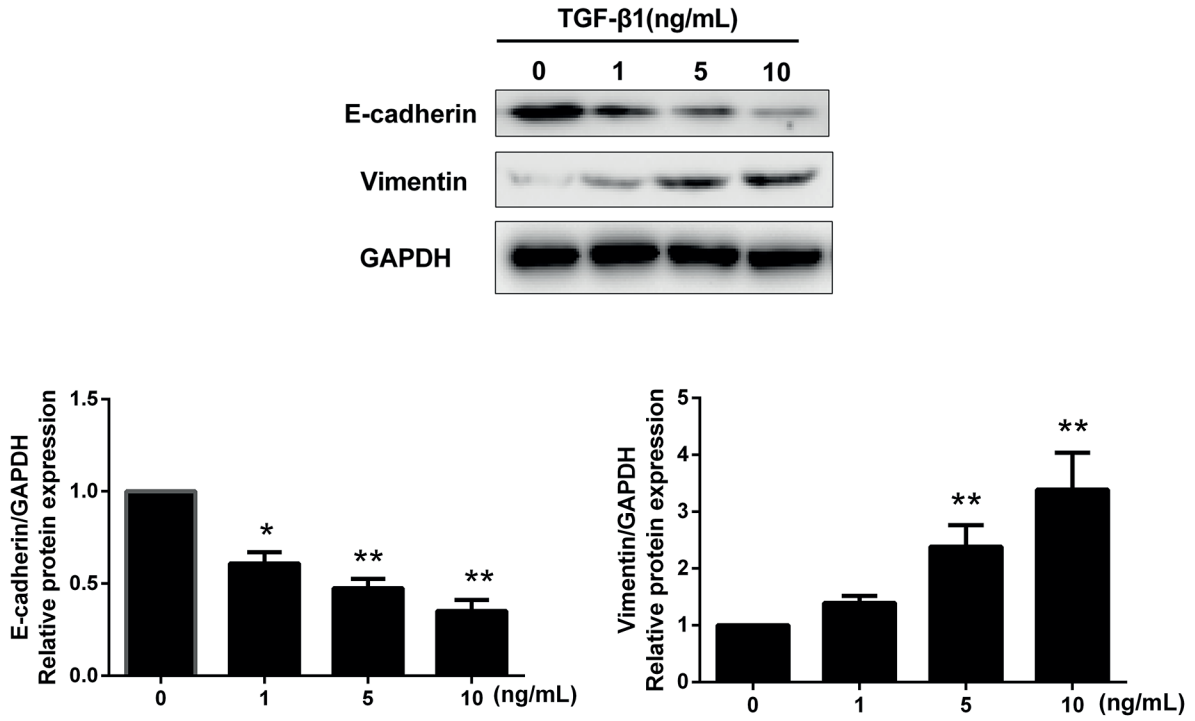


Figure 3. The protein expression level of E-cadherin and Vimentin of Tca8113 cells induced by different concentrations (0 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL) of TGF-β1 for 24 h.

cluding breast cancer, cervical cancer, liver cancer and colon cancer, and the close association between the number of tumor cells with EMT in the tumor tissues and the degree of metastasis and invasion of lesion tissues[11-13]. Greenburg et al[14] found that the epithelial cells in *in-vitro* culture can gradually obtain the characteristics of interstitial cells, and the occurrence of EMT was confirmed. EMT plays an important role in the processes of embryogenesis, organ development, organ and tissue fibrosis as well as the metastasis and invasion of tumor cells. Research showed that snail and Slug, two EMT-related indexes, are highly expressed in the OSCC, but the expression of E-cadherin is low in the cells with epithelial phenotype, and there is a positive correlation between the expression of E-cadherin and the prognosis of OSCC[15-17], confirming that the occurrence and development of OSCC is concomitant with the EMT. As an important member of TGF-β superfamily, TGF-β1 is a kind of cytokine with various biological activities and able to regulate the important physiological processes such as cell proliferation, apoptosis, differentiation and angiogenesis[18]. In the occurrence of EMT, TGF-β1 acts as the major inducer to exert the important effect, which has been widely accepted, and regulates the whole process of occurrence and development of EMT[19,20]. Nevertheless, studies on whether the TGF-β1 can induce the occurrence of EMT in the Tca8113 cells of OSCC have been scarcely reported. Thus, based on the above research results, we inferred that TGF-β1 can induce the occurrence of EMT in the Tca8113 cells of OSCC, resulting in the increased metastasis and invasion, leading to a distant metastasis of tumors.

In this study, we firstly verified whether TGF-β1 could induce the occurrence of EMT in the Tca8113 cells of OSCC, and the results showed that after being treated by 10 ng/mL of TGF-β1 for 24h, most of the cells were significantly stretched like the elongated spindle; cells were transformed from the tightly connected polygonal cobblestone-like distribution into the spindle fibroblast-like distribution with an elongated distance among cells, and the cells growth was dispersive. Such a result suggested that TGF-β1 could induce the occurrence of typical EMT. In the occurrence of EMT, the expression of E-cadherin, the major marker adhered between the epitheliums, was downregulated, while the upregulation of Vimentin, the major interstitial marker, reduced the adhesive force

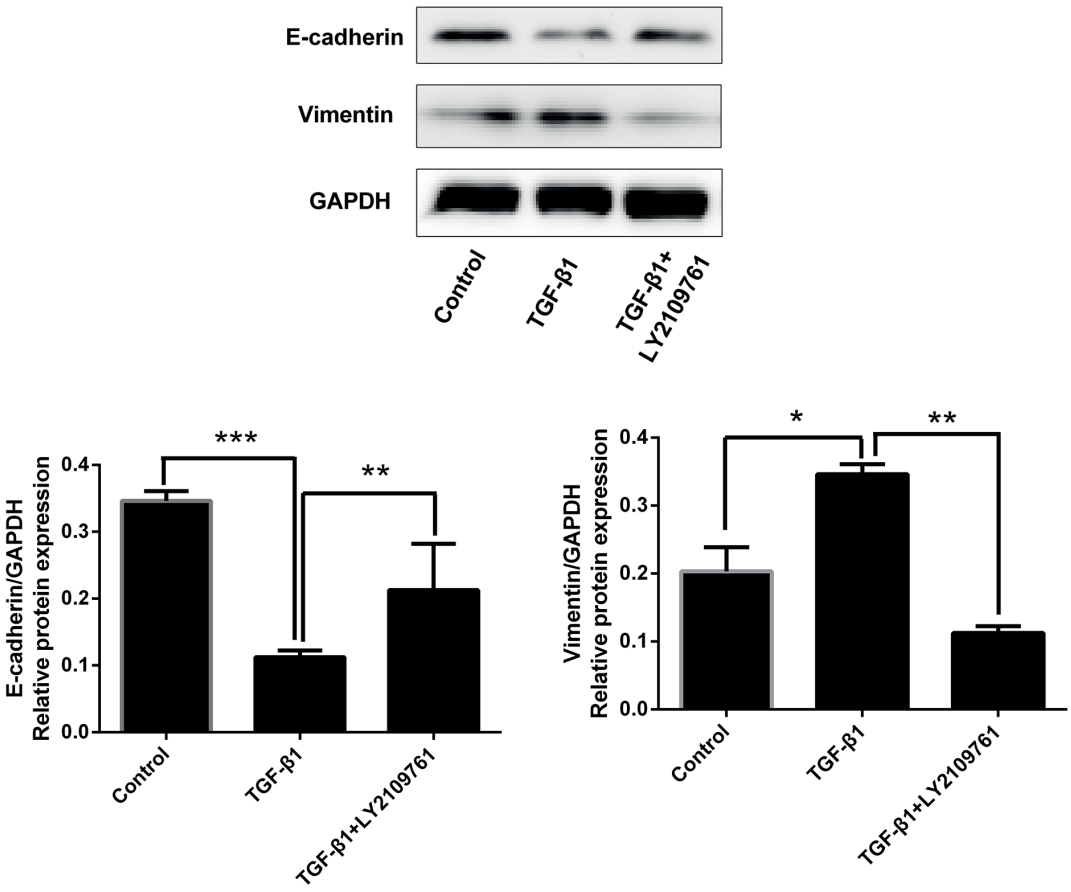


Figure 4. TGF-β1 inhibitor LY2109761 reverse TGF-β1 induced EMT.

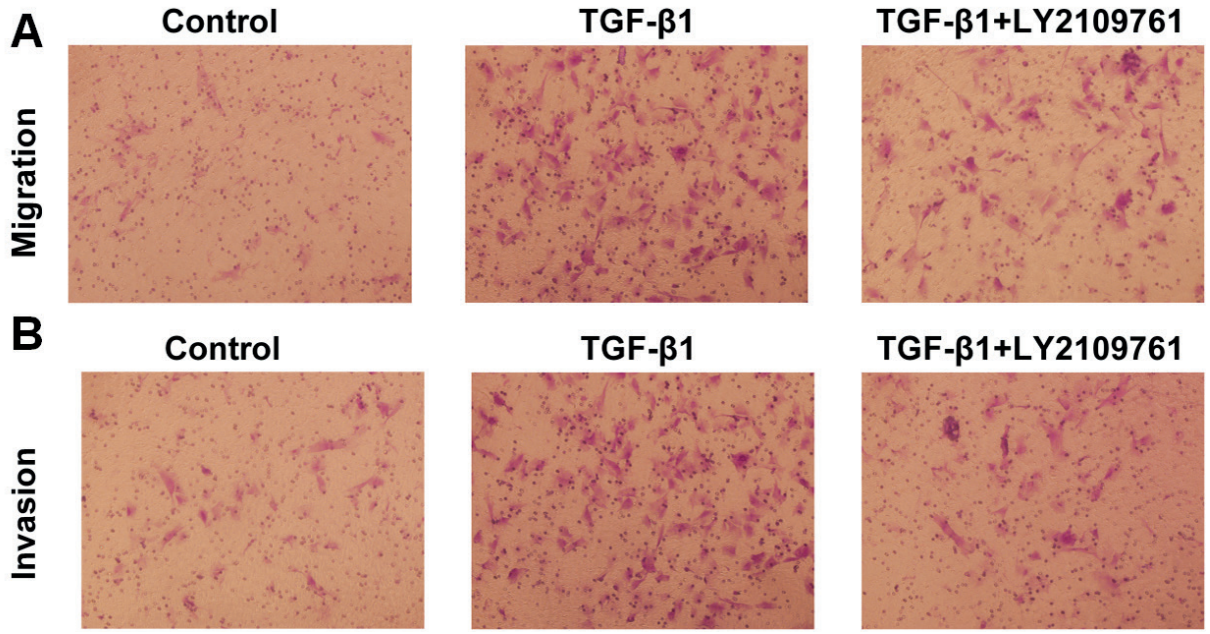


Figure 5. TGF-β1 promotes migration and invasion of Tca8113 cells. A.TGF-β1 promotes migration of Tca8113 cells (crystal violet staining× 200) (crystal violet staining× 200); B.TGF-β1 promotes invasion of Tca8113 cells (crystal violet staining× 200) (crystal violet staining× 200)

among cell. This lead to the increase in migration and invasion of tumor cells, further resulting in infiltration, growth and metastasis of tumors towards the peripheral tissues. Then, through the levels of mRNA and proteins, we further verified that EMT can occur in the Tca8113 cells treated by TGF-β1. After Tca8113 cells were treated with TGF-β1 at various concentrations (0 ng/mL, 1 ng/mL and 5 ng/mL) for 24h, RT-PCR and Western blot indicated that in Tca8113 cells treated with 5 ng/mL of TGF-β1 for 24h, the mRNA and protein expression levels of E-cadherin, the epithelial marker of Tca8113 cells, reached the lowest, but the levels of Vimentin were increased to the maximum. In this process, we only found the upregulated expression of Fibronectin, the interstitial marker, instead of downregulated expression of E-cadherin or upregulated expression of Vimentin. This suggested that TGF-β1 could successfully induce the EMT in the Tca8113 cells of OSCC. Thereafter, based on this verified hypothesis, we conducted the transwell experiment to detect the variations in the ability of migration and invasion of Tca8113 cells. After the cells were treated by 5 ng/mL of TGF-β1 for 24h, the ability of migration and invasion of Tca8113 cells in the TGF-β1 stimulation group was substantially enhanced, which, however, was effectively weakened by the LY2109761, TGF-β1 inhibitor. The result further confirmed that TGF-β1 could enhance the ability of migration and invasion of Tca8113 cells by inducing the EMT.

Conclusions

Our preliminary studies confirm that in the Tca8113 cells of OSCC, TGF-β1 can enhance the ability of migration and invasion by inducing the EMT. This is beneficial for a further understanding about the pathogenesis of OSCC. Additional studies focusing on the effect of EMT in the occurrence and development of OSCC, as well as relevant regulatory mechanism, are required. Our study is important for identifying targets for decreasing the metastasis and invasion of tumor cells by intervening or reversing the EMT process of tumor cells. Thus, the results can provide new ideas as well as a reference for the development of specific treatments for OSCC.

Conflict of interest

The authors declare no conflicts of interest.

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