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Snail/Slug Promote Cell Proliferation of Oral Squamous Cell Carcinoma by Regulating YAP Signal

Zhiwen Xu

Department of Oral and Maxillofacial Head and Neck Surgery, Huashan Hospital Affiliated to Fudan University, Shanghai, 200040, China.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity. Currently, no specific treatment is utilized to prevent the progression of OSCC. This study aimed to clarify whether Snail/Slug promoted OSCC cell proliferation by regulating yes-associated protein 1 (YAP). In this cell culture study, an OSCC cell model was constructed, followed by transfection with Snail-inhibitor. The OSCC HSC-3 cells were treated, and their apoptosis and viability were evaluated. Finally, Western blot, flow cytometry assay, Transwell assay, and scratch assay were used to explore the molecular mechanisms by which Snail/Slug promoted OSCC cell proliferation by regulating YAP. The activation of the Snail/Slug pathway promoted the progression of OSCC. The Snail/Slug regulated the OSCC process via targeting YAP. Inhibition of the Snail/Slug pathway, however, reversed the effect of YAP on OSCC. These findings suggest that the Snail/Slug pathway promoted OSCC progression by targeting YAP.

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INTRODUCTION

Oral squamous cell carcinoma cell (OSCC), which is the most common head and neck squamous cell carcinoma, seriously threatens human health and life (Fan *et al*., 2022; Vitório *et al*., 2020). Although numerous treatment methods have been used to treat OSCC, the five-year survival rate of patients with OSCC is still low (Peng *et al*., 2020; Zhu *et al*., 2020). The main cause of the poor therapeutic effect of OSCC treatments is that the molecular mechanisms underlying the progression of OSCC remains unknown. Interestingly, some studies (Cho *et al*., 2019; Hao *et al*., 2019) have indicated that the Snail/Slug signaling pathway is involved in the development of OSCC (Dantas *et al*., 2021; Jiang *et al*., 2016). However, the detailed mechanisms by which Snail/Slug regulates OSCC is still elusive. Therefore, it is urgent to conduct related study to uncover the mechanisms. Yes-associated protein 1 (YAP) is a well-known main molecule of the Hippo pathway that regulates wound healing, development, tumor, and tissue homeostasis and regeneration (Boopathy and Hong, 2019; Nguyen and Yi, 2019). A clinical trial by Hasegawa *et al*. (2021) pointed out that YAP promoted the development of OSCC. Presently available data have suggested that Snail/Slug and YAP are involved in the progression of OSCC. However, the clear mechanisms of Snail/Slug and YAP are still elusive.

The *Snail/Slug* genes belong to the zinc finger transcription factor superfamily and can inhibit the transcription of downstream target genes by binding to their E-box domain (Lee *et al*., 2020; Malgulwar *et al*., 2018). In multiple cell system studies, it has been found that Snail directly inhibits the transcription of E-cadherin by binding to the E-box promoter, and the Snail/Slug pathway plays a key role in embryonic development, tumor metastasis, and tissue fibrosis processes (Aborisade *et al*., 2022; Zhong *et al*., 2021). A clinical study by Cho *et al*. (2019) has investigated the relationship between the Snail/Slug pathway and OSCC patients, and their results showed that high Snail/Slug expression can promote the progression of OSCC. However, it is unclear whether Snail/Slug regulating downstream targets is involved in the development of OSCC. Interestingly, the regulation of YAP by Snail/Slug participates in the process of numerous diseases. However, the role of Snail/Slug regulating YAP in OSCC is unknown. In this study, we established an *in vitro* model of OSCC to investigate the molecular mechanisms by which Snail/Slug affected OSCC cell proliferation and

* Corresponding author: xu_zhiwen@fudan.edu.cn
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the involvement of YAP in this process.

MATERIALS AND METHODS

Cell culture and treatment

In this cell culture study, the OSCC cell line HSC-3 was obtained from Promo Cell Co., Ltd and cultured in DMEM medium (Thermo Fisher, USA) accompanied by the addition of 1% penicillin/streptomycin and 10% FBS (Biologic Industries), at 37°C and humidified air with 5% CO_2. The OSCC cell line HSC-3 was divided into three groups: Control group, model group and Snail-inhibitor group. OSCC cell line HSC-3 for the model group and Snail-inhibitor group were treated with $CoCl_2$ to establish the OSCC cell model. For the Snail-inhibitor group HSC-3 cells were treated with Snail-inhibitor (constructed by Shanghai GeneChem Co., Ltd.), while HSC-3 cells in control group were treated with PBS.

Western blotting

The proteins extracted from HSC-3 cells was subjected to 10% SDS-PAGE and loaded, transferred to a PVDF membrane, and washed with TBST for 5 min. A primary antibody (1:2,000; Bioworld Techmology, Inc, China) and GAPDH (1:1,000; Bioworld Techmology, Inc, China) were added and incubated overnight at 4 °C. After washing with TBST three times (10min/time), a secondary antibody (1:10,000; Bioworld Techmology, Inc, China) was added and blocked for 2 h at room temperature. After washing with TBST three times (10min/time), ECL luminescent reagent was added for development, and the gray value of the bands were analyzed.

Flow cytometry assay

The apoptosis was analyzed by flow cytometry after cell treatment. Briefly, cells were collected and stained with Annexin V-FITC and Propidium Iodide (PI) in the darkness according to the apoptosis detection kit (C1062, Beyotime) procedure. After treatment, cells in all groups were analyzed by a flow cytometer (NovoCyte, Aceabio, San Diego, CA, USA).

Transwell assay

The matrix adhesive was diluted in a ratio of about 1:8 using serum-free medium on ice. Serum-free medium was added to the upper chamber, covering the bottom, and the chamber was then incubated in a cell incubator for 1-4 h. Following this, 200 µL cell suspensions were added, and a volume of 500 µL complete culture medium was added to the lower chamber. Care was taken not to generate bubbles when returning to the chamber. The Transwell board was returned to the incubator, and cultivation continued for 24-48 h.

Afterwards, the culture medium from the upper and lower chambers was discarded, and the upper and lower chambers were cleaned three times with PBS. Un-invasive cells in the upper chamber were wiped off with a cotton swab. Polyformaldehyde (500 µL) was added to the lower chamber, placed in a small room, and fixed for 30 min. The polyformaldehyde was then discarded, and the chamber was air-dried. Subsequently, 500 µL crystal violet solution was added to the lower chamber, left to stain for 30 min, and then the crystal violet solution was discarded. The staining solution was cleaned with PBS, the chamber was dried, and observations and photos were taken under a microscope.

Scratch assay

The original culture medium was aspirated, and the cells were cleaned with PBS. A volume of 1 mL pancreatic enzyme digestion solution was added to digest the cells. The cell suspension was collected in a centrifuge tube and centrifuged at room temperature for 5 min at 800-1000 rpm/min. Approximately 5×10^5 cells per well were inoculated onto a culture plate, and a scratch was made on the plate using a 20 µL tip. The width of the scratch was observed under a microscope, and photos were taken at 0 and 24 h. Subsequently, the photos were imported into the software for analysis.

Statistical analysis

Prism 8 (GraphPad Software, San Diego, CA, United States) was used to analyze the results. All data were presented as mean±SD from at least three independent experiments. A *t*-test and one-way ANOVA test were utilized for multiple groups comparison, and a p-value < 0.05 indicated statistically significant.

RESULTS

Western blotting was utilized to assess the protein levels of Snai, Slug, and YAP. The results showed that expression levels of Snai, Slug, and YAP proteins in the model group were significantly higher than those in the control and Snail-inhibitor groups (all $P<0.05$) (Fig. 1). These data suggest that the upregulation of Snai, Slug, and YAP proteins promotes the development of OSCC.

The apoptosis level was determined using flow cytometry. The results showed that the apoptotic level of control cells was obviously lower than that of the model group. The apoptotic level of the Snail-inhibitor group was obviously lower than that of the model group (both $P<0.05$) (Fig. 2).

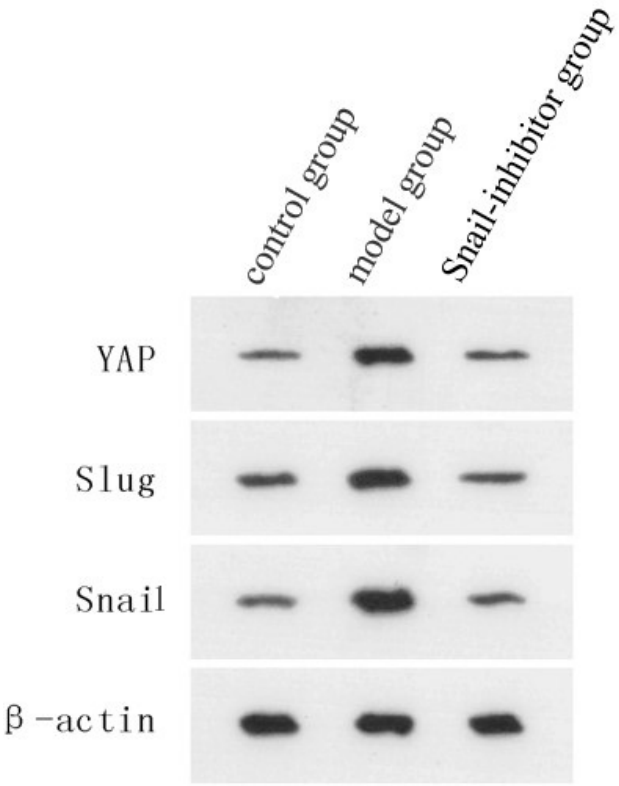

Fig. 1. The expression levels of apoptosis-related protein, including Snail, Slug, and YAP, were determined by Western blotting.

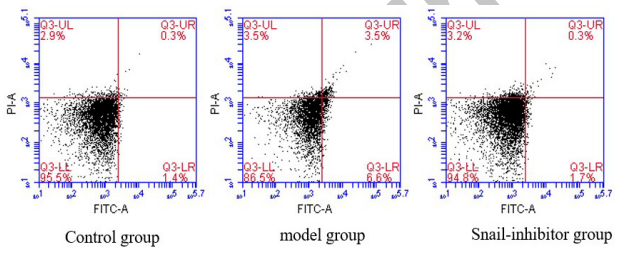

Fig. 2. The apoptotic level of OSCC cells was detected by flow cytometry.

To evaluate OSCC cell invasion and migration, scratch assay was performed. The results showed that the invasion and migration of the control, model, and Snail-inhibitor groups were similar at 0 h. However, the invasion and migration of the control and Snail-inhibitor groups at 24 h were obviously lower than those of the model group (P<0.05) (Fig. 3A).

To evaluate OSCC cell invasion and migration, Transwell assay was performed. The results showed that the invasion and migration of the control and Snail-inhibitor groups were obviously lower than those of the model group (P<0.05) (Fig. 3B).

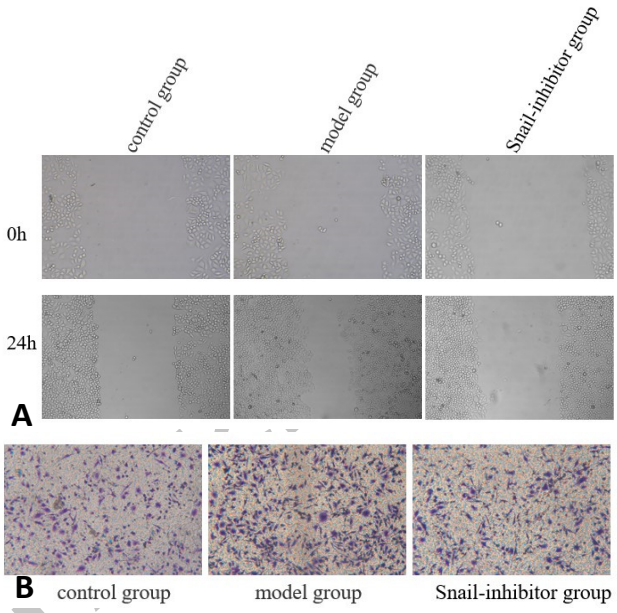

Fig. 3. Cell invasion and migration were detected using scratch assay (A) and Transwell assay (B).

DISCUSSION

The five-year survival rate of OSCC patients is still low at 50%. OSCC remains the leading death cause of oral cancers and had a serious harm on human (Johnson *et al*., 2020; Kitamura *et al*., 2020). Currently, numerous therapeutic methods have been used to treat OSCC. However, OSCC cannot be cured, and only related symptoms were prevented, and complex pathogenesis of OSCC contributed to poor therapeutic effect (Botha *et al*., 2021; Manzano-Moreno *et al*., 2021). Snail/Slug is well-known as distinct the zinc finger transcription factor and was associated in the OSCC process (Chien *et al*., 2019). More and more studies have indicated that Snail/Slug and YAP are involved in the OSCC process, responding studies of these targets in OSCC were still limited and the detailed mechanism of OSCC is still elusive. More trials should be carried out to clarify the mechanism of OSCC. These results in present study mainly investigate the molecular mechanisms by which Snail/Slug promoted OSCC cell proliferation via regulating YAP. These outcomes of our study showed that the activation of Snail/Slug pathway promoted the progression of OSCC. The Snail/Slug pathway regulated the progression of OSCC process via targeting YAP.

Snail/Slug pathway is a zinc finger transcription

factor associated with cancer process by controlling the downstream targets. few studies have investigated the effect of Snail/Slug pathway in OSCC. The present study indicated that activation of Snail/Slug promoted the development of OSCC. Snail/Slug plays a crucial role in cancer and is involved in numerous diseases. Our results suggested that activation of Snail/Slug pathway can promote the development of OSCC, while inhabitation of Snail/Slug pathway can alleviate the OSCC progress. These results were firstly reported and provided potential targets of treating OSCC. A study by Cho *et al*. (2019) points out the role of Snail/Slug expression in OSCC, and conclude that expression of Twist and Snail/Slug, is related to p16 expression in OPSCC, and these results are consistent with those reported by present study.

YAP is well-known as main molecule of Hippo pathway and primarily participated in the development of cancer, and numerous studies reported that upregulation of YAP contributed to cancer development. Currently, the detailed mechanism of YAP in OSCC has not been investigated. Our results have investigated the role of YAP in OSCC, and the results showed that down-regulation of YAP alleviated OSCC, while upregulation of YAP expression can promote the development of OSCC. These results suggest that the YAP/PIEZO1 axis promotes OSCC cell growth. Our results are consistent with those reported by Hasegawa *et al*. (2021).

A limitation of present study was that we only conducted *in vitro* study to investigate that the molecular mechanisms of Snail/Slug promoted OSCC cell proliferation by regulating YAP, and further clinical study was needed to manage.

In conclusion, the current study has clarified the activation of Snail/Slug pathway can promote the progression of OSCC. The Snail/Slug pathway regulates the OSCC development via targeting YAP, and these potential targets would be of value in alleviating OSCC progression.

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IRB approval

The ethic approval was obtained from the Ethic Committee of Huashan Hospital Affiliated to Fudan University.

Ethical statement

The ethic approval was obtained from the Ethic Committee of Huashan Hospital Affiliated to Fudan University.

Data availability

The data are free access to available upon the corresponding author request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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