

## ENHANCED EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA ONE (TGF- $\beta_1$ ) DURING PROGRESSION OF ORAL EPITHELIAL DYSPLASIA TO CARCINOMA

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### **ABSTRACT**

**Objective:** transforming growth factors (TGFs), the pro-inflammatory cytokines play an important role in malignancy. This study was aimed to evaluate immunohistochemically the expression of TGF- $\beta_1$  protein in epithelial dysplasia (ED), carcinoma in situ (CIS) and oral squamous cell carcinoma (OSCC) in order to elucidate its role in oral carcinogenesis.

**Material and methods:** 30 cases of OSCC was investigated including well, moderately and poorly differentiated OSCC (10 cases each) that contained normal epithelium, ED and foci of CIS. They were subjected to immunohistochemistry using antibodies against TGF- $\beta_1$ .

**Results:** the expression of TGF- $\beta_1$  was increased in intensity and distribution in different stages of ED and OSCC when compared to normal mucosa. In ED, TGF- $\beta_1$  intensity score was weak (1+) in 8 cases (26.6%), moderate (2+) in 20 cases (66.6%) and strong (3+) in 2 cases (6.6%). In CIS, the intensity score of TGF- $\beta_1$  was weak (1+) in 3 cases (10%), moderate (2+) in 17 cases (56.6%) and strong (3+) in 10 cases (33.3%). TGF- $\beta_1$  expression was markedly enhanced in both OSCC cells and stromal cells. TGF- $\beta_1$  score of intensity was weak (1+) in 2 cases (20%) of well differentiated and in 1 case (10%) of moderately and poorly differentiated OSCC. TGF- $\beta_1$  score of intensity was moderate (2+) in 3 cases (30%) of well differentiated and moderately differentiated, and in 1 case (10%) of poorly differentiated. On the other hand, the expression score of TGF- $\beta_1$  was strongly positive (3+) in 5 (50%), 6 (60%) and 8 (80%) cases of well, moderately and poorly differentiated OSCC respectively.

**Conclusion:** enhanced expression of TGF- $\beta_1$  could be responsible for transformation of oral premalignant lesions to OSCC as well as more aggressive tumor growth, metastasis and resistance to treatment.

**KEYWORDS:** TGF- $\beta_1$ , epithelial dysplasia, carcinoma in situ, oral squamous cell carcinoma

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## INTRODUCTION

It is estimated that more than 90% of oral malignancies are squamous cell carcinoma (SCC) that arises from dysplastic surface epithelium<sup>1</sup>. Oral squamous cell carcinoma (OSCC) is characterized by a high degree of local invasiveness and a high rate of metastasis to regional cervical lymph nodes<sup>2</sup>.

The high rate of relapse in OSCC indicates the insufficiency of current prognostic predictors in predicting metastatic potential and the tumor outcomes<sup>3</sup>. Despite advances in treatment and the understanding of molecular mechanisms of OSCC, survival rates have not improved significantly<sup>1</sup>.

Transforming growth factor- $\beta$  (TGF- $\beta$ ), one of the cytokines responsible for regulation of cell behavior, is involved in many functions such as cell proliferation, differentiation and extracellular matrix production<sup>4</sup>.

The roles of different TGF- $\beta$  isoforms (TGF- $\beta_1$ , TGF- $\beta_2$ , TGF- $\beta_3$ ) depend on the type, differentiation state and physiological conditions of target cells<sup>5,6</sup>.

It is known that TGF- $\beta$  can function both as a tumor suppressor or tumor promoter, depending on the stage of carcinogenesis<sup>7</sup>. TGF- $\beta$  expression has been implicated in pathogenesis of a variety of diseases including cancer and fibrosis<sup>8</sup>. Moreover, TGF- $\beta_1$  is one of the main epithelial-mesenchymal transition (EMT)-inducing factors in both physiologic and pathologic conditions<sup>9</sup>. In addition, it was demonstrated that both TGF- $\beta_1$  and TGF- $\beta_2$  can function as tumor promoter in early human SCC in a study utilized transfected human malignant epidermal keratinocytes<sup>10</sup>.

Few studies, however, have been conducted to explore the exact role of TGF- $\beta$  in oral epithelial dysplasia and OSCC. Therefore, in the present study, we aimed to investigate the expression pattern of TGF- $\beta_1$  in dysplastic oral epithelium and OSCC to illustrate the potential role of TGF- $\beta_1$  in progression of OSCC from epithelial dysplasia.

## MATERIALS AND METHODS

### Selection of cases

Thirty cases of oral squamous cell carcinoma (OSCC) were retrieved from archival pathology files of Department of Oral Pathology, Faculty of Dentistry, Tanta University during the last ten years from January 2005 to December 2014. These cases included well differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC (10 cases each) that simultaneously contained normal epithelium, dysplastic epithelium and foci of carcinoma in situ (CIS). Their diagnosis was based on the clinical and histopathological examination. The experimental protocol for analyzing surgical materials was approved by the Research Ethics Committee of Faculty of Dentistry, Tanta University. The tissue specimens were all surgical materials and were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Serial sections were cut at 5  $\mu$ m thickness; one of each set of sections was stained with haematoxylin and eosin (HE). Another set was immunohistochemically stained for TGF- $\beta_1$ .

### Antibodies

Rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of TGF- $\beta_1$  [(V): sc-146] of human origin was obtained from Santa Cruz Biotechnology, Inc.

### Immunohistochemistry

Immunohistochemistry was performed using the ChemMate Envision<sup>TM</sup> system (Dako) as described elsewhere<sup>11</sup>. For TGF- $\beta_1$ , sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity and incubated with 5% milk protein in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Triton X-100 (T-PBS) for 1 hour at room temperature to block non-specific protein binding sites. They were then incubated overnight at 4°C with the primary antibodies diluted at 1:50 (anti- TGF- $\beta_1$ ) in PBS. After overnight incubation,

the sections were incubated with the Envision reagents for 1 hour at room temperature and treated with 0.02% 3,3'-diaminobenzimine in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide to visualize the reaction products<sup>12</sup>. Finally, the sections were counterstained with Dako REAL™ Hematoxylin (Dako). For negative control, the primary antibodies were replaced with preimmune IgGs.

#### Analysis of TGF- $\beta_1$ staining

The positive result for TGF- $\beta_1$  in normal control, dysplasia, CIS and OSCC was indicated by the development of yellow to brown cytoplasmic or cytoplasmic and nuclear precipitates, detected by a light microscope. This involved the comparison of staining intensity of the tumor cells and the number of positive cells in proportion to the total number of tumor cells in a given tumor areas scored upon microscopic examination. The protein expression was analyzed using a semiquantitative validated scoring method<sup>13</sup>. The mean percentage of positive

cells was determined in five microscopic areas at a magnification of  $\times 400$ , as the following groups: score 3+ (strongly positive); when positive cells  $>50\%$  of the total cells, score 2+ (moderately positive); positive cells  $>20\%$  but  $<50\%$ , score 1+ (weakly positive); positive cells  $>10\%$  but  $<20\%$ , and score 0 (negative); positive cells  $<10\%$ .

#### RESULTS

##### *TGF- $\beta_1$ Localization in Normal Mucosa, ED and CIS*

In normal epithelia obtained simultaneously with squamous cell carcinoma, TGF- $\beta_1$  exhibited low expression pattern (Fig. 1a, d) mainly in basal and parabasal cells. In epithelial dysplasia, where the criteria of dysplastic changes were seen in the lower half of the epithelium and are characterized by nuclear hyperchromatism, pleomorphism and cellular crowding (Fig. 1b), the expression of TGF- $\beta_1$  was cytoplasmic and expressed sporadically in lower third and in some sporadic prickle cells (Fig. 1e). In CIS where the dysplastic changes extend

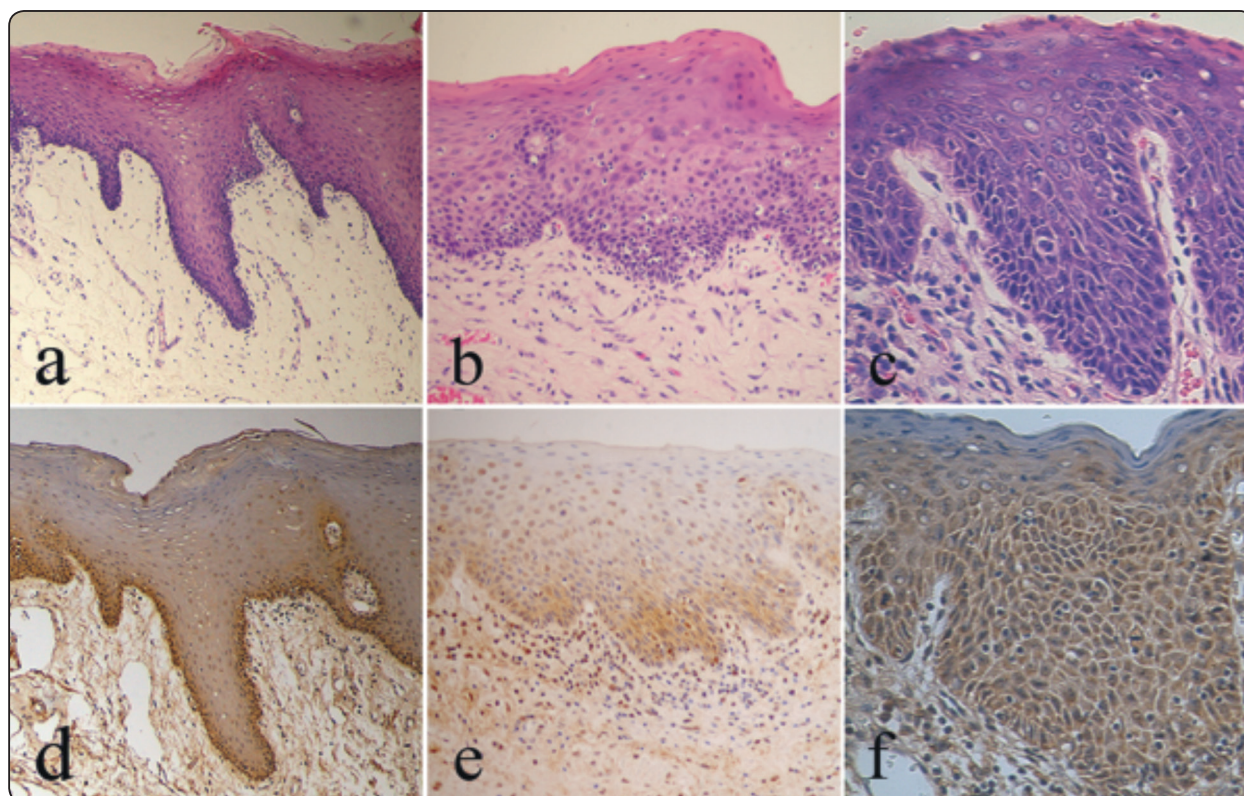


Fig. (1) TGF- $\beta_1$  expression in normal, dysplastic epithelia and CIS.

throughout the entire thickness of epithelium but the basement membrane still intact (Fig. 1c), a strong cytoplasmic expression of TGF- $\beta_1$  was observed involving the whole thickness of epithelium (Fig. 1f). Low to moderate cytoplasmic expression of TGF- $\beta_1$  was localized in few numbers of cells of the underlying connective tissue in ED whereas it was strong and diffuse in CIS. The score of intensity of TGF- $\beta_1$  was weak (+) in 28 cases of normal mucosa, 8 cases (26.6%) in ED and 3 cases (10%) of CIS. The expression score of TGF- $\beta_1$  was moderate (++) in 2 cases of normal mucosa, 20 cases (66.6%) in ED and 17 cases (56.6%) of CIS. On the other hand, the expression score of TGF- $\beta_1$  was strong (+++) in 2 cases (6.6%) of ED and 10 cases (33.3%) of CIS (table1, Fig 2).

(HE) (a, c and e) and immunoperoxidase stains for TGF- $\beta_1$  (b, d and f), hematoxylin counterstain. In normal epithelia, expression of TGF- $\beta_1$  is mainly in basal and parabasal cells (d). In epithelial dysplasia, the expression of TGF- $\beta_1$  is cytoplasmic and expressed in lower third and in some sporadic prickles cells (e). In CIS, there is a strong cytoplasmic expression of TGF- $\beta_1$  involves the whole thickness of epithelium (f). Expression of TGF- $\beta_1$  is localized in few numbers of cells of the underlying connective tissue in epithelial dysplasia whereas it is strong and diffuse in CIS. (a-e)  $\times 100$ ; (f)  $\times 200$ .

#### TGF- $\beta_1$ localization in the parenchyma of oral squamous cell carcinoma

In well differentiated squamous cell carcinoma (Fig. 2a), TGF- $\beta_1$  was strongly expressed in the nucleus and cytoplasm in the peripheral tumor cells and not positive in central keratinized cells (Fig. 2b). In moderately differentiated squamous cell carcinoma (Fig. 2c), TGF- $\beta_1$  was also strongly expressed in almost all malignant cells in nuclear and cytoplasmic fashions (Fig. 2d). In poorly differentiated squamous cell carcinoma (Fig. 2e) where there are scattered malignant cells with nuclear pleomorphism and without keratin formation, TGF- $\beta_1$  expression was markedly

TABLE (1) TGF- $\beta_1$  staining score of intensity in various oral epithelial carcinogenesis

Types of oral epithelia (No.)	Score of Intensity			Total
	+	++	+++	
Normal epithelia	28	2	–	30
Dysplastic epithelia	8	20	2	30
CIS epithelia	3	17	10	30
well differentiated OSCC	2	3	5	10
Moderately differentiated OSCC	1	3	6	10
Poorly differentiated OSCC	1	1	8	10

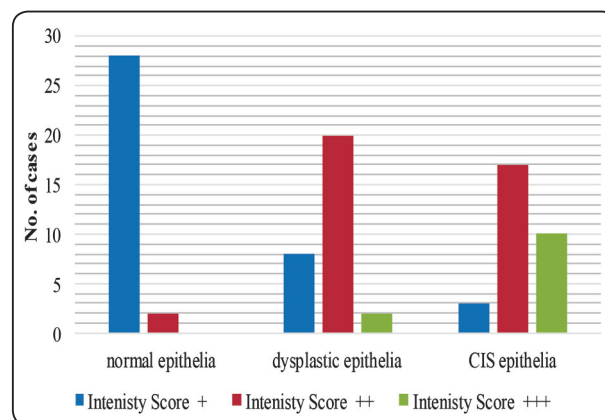


Fig. (2) Graph represents the TGF- $\beta_1$  staining score of intensity in different oral epithelial dysplasia

enhanced in the nucleus and cytoplasm of OSCC cells (Fig. 2f). There was strong expression in the stromal cells in different grades of OSCC (Fig. 2b, d, f). The score of intensity of TGF- $\beta_1$  was weak (+) in 2 (20%) cases of well differentiated and in 1 (10%) case of moderately and poorly differentiated SCC. The expression score of TGF- $\beta_1$  was moderate (++) in 3 (30%) cases of well differentiated and moderate differentiated, and in 1 (10%) case of poorly differentiated SCC. On the other hand, the expression score of TGF- $\beta_1$  was strongly positive (+++) in 5 (50%), 6 (60%) and 8 (8%) cases of well, moderate and poorly differentiated SCC respectively. (table1, Fig 4).



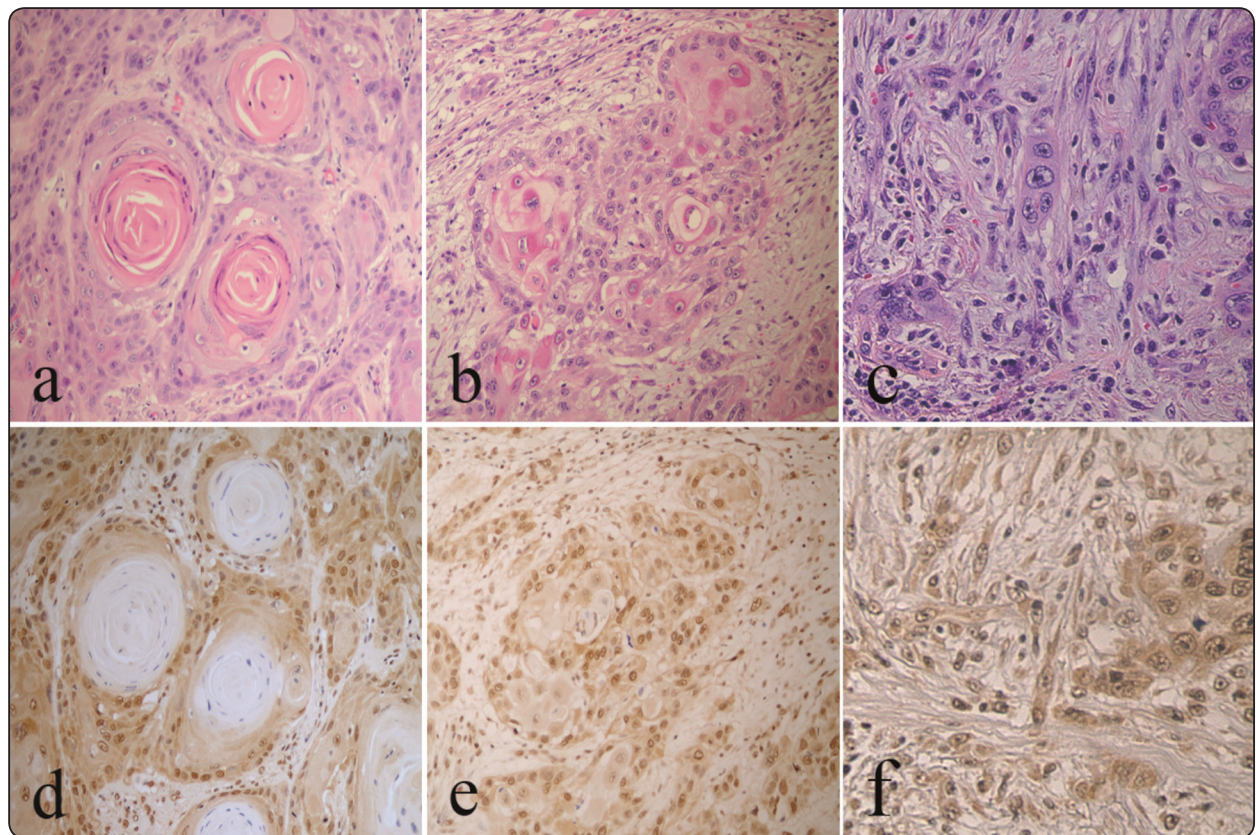


Fig. (3) Immunohistochemical profiles for TGF- $\beta_1$  in three grades of oral squamous cell carcinoma. HE (a, c and e) and immunoperoxidase stains for TGF- $\beta_1$  (b, d and f), HE. In well differentiated SCC, TGF- $\beta_1$  is strongly expressed in peripheral cells but not expressed in central keratinized cancer cells (d). In moderately differentiated squamous SCC, TGF- $\beta_1$  is strongly expressed in almost all malignant cells in nuclear and cytoplasmic fashions (e). In poorly differentiated SCC (e), TGF- $\beta_1$  expression is markedly enhanced (f). There is a positive expression of TGF- $\beta_1$  in the stromal cells. (a-d)  $\times 100$ ; (e, f)  $\times 400$ .

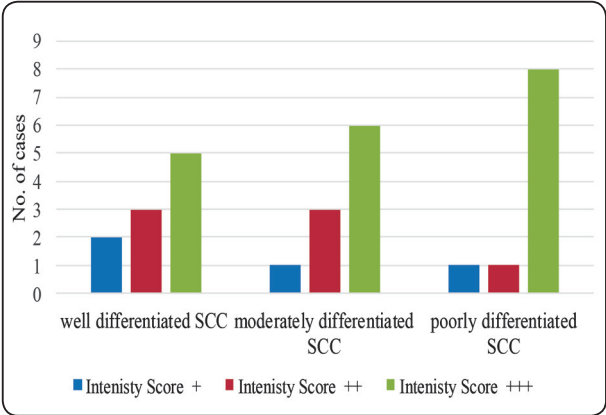


Fig. (4) Graph represents the TGF- $\beta_1$  staining score of intensity in different grades of OSCC.

# DISCUSSION

The present study investigated the TGF- $\beta_1$  protein expression pattern in 30 cases of OSCC and associated ED and CIS to elucidate its possible role in carcinogenesis of oral mucosa as well as during the progression from mild epithelial dysplasia to the CIS and to OSCC including well, moderately and poorly differentiated. Premalignant oral lesions have a high incidence of recurrence and progression to malignant disease. However, despite studies showing the contribution of TGF- $\beta_1$  to cancer progression, there is controversy as regard to its expression in premalignant and malignant lesions<sup>14-24</sup>.

This study demonstrated that the intensity and distribution of TGF- $\beta_1$  protein expression was increased from normal mucosa- mild epithelial dysplasia- CIS sequence. Moreover, the expression was up regulated from moderately to poorly differentiated OSCC. These findings were in accordance with the previous studies conducted on premalignant and malignant lesions involving the oral mucosa and other body organs such as cervix, lung, liver, esophagus<sup>14-21</sup>. However, on contrary to the present study, numbers of reports demonstrated the down regulation of TGF- $\beta_1$  expression in premalignant lesion when compared with normal mucosa and in poorly differentiated OSCC in comparison with well and moderately differentiated OSCC<sup>22-24</sup>. These controversial results may be as previously suggested attributed to the dual role of TGF- $\beta_1$  in carcinogenesis which is extremely a complex process, arises from the fact that the actions of the ligand are context dependent and because the differences between the TGF- $\beta_1$  isoforms have been documented<sup>26,27</sup>. Added to that TGF- $\beta_1$  can function both as a tumor suppressor or tumor promoter, depending upon the cellular context and / or the stage of carcinogenesis<sup>6,27</sup>.

The molecular mechanisms that underlie the switch of TGF- $\beta$  from tumor suppressor to promoter are still largely unclear and need to be confirmed<sup>10,28</sup>. In normal physiological conditions, TGF- $\beta_1$  act as a potent growth inhibitor and has tumor-suppressing activity. Loss of the growth inhibitory response to TGF- $\beta_1$  is a common feature of epithelial cancers<sup>25-27</sup>. This seems to apply also to early tumor stages. However, during subsequent tumor progression, TGF- $\beta_1$  secreted by tumor cells and stromal cell contributes to tumor invasion and metastasis by paracrine and autocrine stimulation<sup>28</sup>.

The expression of TGF- $\beta_1$  in the present study was also enhanced in stromal cells of OSCC in agreement with the previous reports, which found that

TGF- $\beta_1$  protein was expressed in stromal tissues of noncancerous squamous epithelium and esophageal SCC with intratumoral vascularity. Additionally, TGF- $\beta_1$  knockdown in fibroblasts reduced migration and invasive abilities in cocultured TE8 cells<sup>29</sup>. One of previous studies which support the present study reported that TGF- $\beta$  stimulates motility of premalignant lesion cells and up regulates expression of paxillin, as well as its co-localization with Protein Phosphatase-1 which is an intermediate in the TGF- $\beta$ -mediated regulation of premalignant lesion cell motility, while concurrently diminishing the level of paxillin serine phosphorylation<sup>30</sup>. Head and neck squamous cell carcinoma (HNSCC) tumor secretion of TGF- $\beta$  function to directly inhibit cytotoxic T cell-mediated immunity and to recruit to the tumor site additional immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs), the less mature CD34+ progenitor cells, as well as M2-skewed macrophages<sup>31</sup>. In the present study, the overexpression of TGF- $\beta_1$  in adjacent premalignant tissues to the OSCC from the same patient is suggesting that its overexpression is an early event during OSCC development as reported in HNSCC<sup>21</sup>.

Targeting TGF- $\beta$  signaling pathway for the treatment of several human malignancies include the use of neutralizing antibodies, antisense oligonucleotides and small molecule inhibitors of kinase activity of the TGF- $\beta$  receptor complex could be a very promising therapeutic approach.<sup>32,33</sup>

In conclusion, overexpression of TGF- $\beta_1$  could be responsible for transformation of oral premalignant lesions to OSCC as well as more aggressive tumor growth, metastasis and resistance to treatment. Further studies at the molecular level is necessary to clarify the exact molecular mechanisms underlying the role of TGF- $\beta_1$  in oral cancer, which would be beneficial in identifying novel targets for therapeutic intervention.

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